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HEME OXYGENASE-1 IN CARDIOVASCULAR DISEASES

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ACADEMIC DISSERTATION

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To my family

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ORIGINAL PUBLICATIONS

ABBREVIATIONS

AF	Aortic flow
ANOVA	One way analysis of variance
ANP	Atrial natriuretic peptide
APACHE II	Acute Physiology and Chronic Health Evaluation II score
AUC	Area under curve
CF	Coronary flow
cGMP	Guanosine 3'5'-cyclic monophosphate, cyclic GMP
CO	Carbon monoxide
COHb	Carboxyhemoglobin
Coll1a1	Prococollagen type I alpha 1
Coll3a1	Prococollagen type III alpha 1
CoPPIX	Cobalt protoporphyrin IX
CORM	Carbon monoxide-releasing molecule
CRP	C-reactive protein
CSC	Cardiac stem cell
CTGF	Connective tissue growth factor
cTnI	Cardiac troponin I
Cx43	Connexin 43
DAPI	4´6-diamino-2-phenylindole
+/- dp/dt	Positive and negative first derivative of left ventricular pressure
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-regulated kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H3P	Phosphorylated histone H3
HIF-1α	Hypoxia-inducible factor 1 alpha
HO	Heme oxygenase
HR	Heart rate
ICD-10	International Classification of Diseases 10th edition
ICU	Intensive care unit
IL	Interleukin
IQR	Interquartile range
I/R	Ischemia/reperfusion
LAD	Left anterior descending coronary artery
LD	Linkage disequilibrium
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end-diastolic pressure
MAPK	Mitogen-activated protein kinase
MC	Methylene chloride

MHC	Cardiac myosin heavy chain
MI	Myocardial infarction
MOD	Multiple organ dysfunction
MSC	Mesenchymal stem cell
NO	Nitric oxide
Nrf2	Nuclear factor-erythroid 2-related factor 2
PAI-1	Plasminogen activator inhibitor type 1
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
ROC	Receiver-operating characteristic
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SAPS II	Simplified Acute Physiology Score II
SDF-1α	Stromal cell-derived factor 1 alpha
SEM	Standard error of mean
sGC	Soluble guanylate cyclase
SMA	Smooth muscle actin
SMC	Smooth muscle cell
SNP	Single-nucleotide polymorphism
SOFA	Sequential Organ Failure Assessment score
Tbx18	T-box transcription factor Tbx18
TGF-β1	Transforming growth factor beta1
TNF-α	Tumor necrosis factor α
TRITC	Tetramethyl rhodamine isothiocyanate
TUNEL	Terminal transferase-mediated DNA nick-end labeling
VEGF	Vascular endothelial growth factor
VF	Ventricular fibrillation
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor
ZnPP IX	Zinc protoporphyrin IX

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals (I-V). In addition, some unpublished data are presented.

I Lakkisto P, Palojoki E, Bäcklund T, Saraste A, Tikkanen I, Voipio-Pulkki L-M, Pulkki K. Expression of Heme Oxygenase-1 in Response to Myocardial Infarction in Rats. J Mol Cell Cardiol 2002; 34: 1357-1365.

II Lakkisto P, Kytö V, Forsten H, Siren J-M, Segersvärd H, Voipio-Pulkki L-M, Laine M, Pulkki K, Tikkanen I. Heme oxygenase-1 and carbon monoxide promote neovascularization after myocardial infarction by modulating the expression of HIF-1α, SDF-1α and VEGF-B. Eur J Pharmacol 2010; 635: 156-164.

III Lakkisto P, Siren J-M, Kytö V, Forsten H, Laine M, Pulkki K, Tikkanen I. Heme oxygenase-1 induction protects the heart and modulates cellular and extracellular remodeling after myocardial infarction in rats. Submitted.

IV Lakkisto P, Csonka C, Fodor G, Bencsik P, Voipio-Pulkki L-M, Ferdinandy P, Pulkki K. The heme oxygenase inducer hemin protects against cardiac dysfunction and ventricular fibrillation in ischemic/ reperfused rat hearts: role of Cx43. Scand J Clin Lab Invest 2009; 69: 209-218.

V Saukkonen K*, Lakkisto P*, Kaunisto M, Varpula M, Voipio-Pulkki L-M, Varpula T, Pettilä V, Pulkki K. Heme oxygenase-1 polymorphism and plasma concentrations in the critically ill patients. Shock 2010; published online April 6; doi: 10.1097/SHK.0b013e3181e14de9.
* Equal contribution

The publication V has been included in the thesis for doctoral degree by Katri Saukkonen at University of Helsinki (Helsinki, Finland, 2010).

ABSTRACT

Myocardial infarction (MI) is a major cause of morbidity and mortality. MI leads to left ventricular remodeling, which may eventually progress to heart failure. New strategies are needed for protecting the myocardium against ischemic injury and enhancing the recovery and repair of the infarcted heart. The present studies were undertaken to investigate the role of heme oxygenase-1 (HO-1) in protection and repair of infarcted and ischemic/reperfused hearts and to examine the potential mechanisms mediating the protective effects of HO-1 in these experimental rat models. In addition, the role of *HO-1* polymorphisms and HO-1 plasma concentrations in critically ill patients and in the subgroup of cardiac patients was evaluated.

A total of 334 adult male Wistar rats were used in the experimental studies (278 rats in I–III and 56 in IV). An experimental MI model was used to investigate the expression and localization of HO-1 in the infarcted hearts and to assess the effects of HO-1 induction and carbon monoxide (CO) donor pretreatment on recovery and regeneration of the infarcted hearts (I–III). Gene expression was measured by real-time RT-PCR and protein levels by Western blotting and ELISA. Immunohistochemical analysis was used to assess cardiac regeneration and ventricular remodeling in the infarcted rat hearts. Isolated rat heart preparations were used to investigate the protective effect of HO-1 against ischemia/reperfusion (I/R)-induced cardiac dysfunction and ventricular arrhythmias (IV). HO-1 plasma concentrations and *HO-1* polymorphisms were assessed in 231 critically ill intensive care unit (ICU) patients (154 men and 77 women, 17–87 years of age), and the association of *HO-1* polymorphisms and plasma levels with illness severity, organ dysfunction, ICU, and hospital mortalities was examined (V). Illness severity was determined by the Simplified Acute Physiology Score (SAPS) II and Acute Physiology and Chronic Health Evaluation (APACHE) II score, and the degree of organ dysfunction by the Sequential Organ Failure Assessment (SOFA) score.

In the experimental studies, HO-1 expression was induced in the infarcted rat hearts, especially in the infarct and infarct border areas (I–III). HO-1 protein was localized in the vascular walls, the cardiomyocytes of the infarct border area, and the monocytes/macrophages and fibroblasts of the infarct area. HO-1 induction and CO donor pretreatment had differential effects on the infarcted rat hearts. They both promoted neovascularization in the infarcted hearts, but CO activated c-kit+ stem/progenitor cells via hypoxia-inducible factor 1α (HIF-1α), stromal cell-derived factor 1α (SDF-1α), and vascular endothelial growth factor B (VEGF-B), and promoted vasculogenesis and formation of new cardiomyocytes, whereas HO-1 promoted angiogenesis possibly via SDF-1α. In addition, HO-1 had many beneficial effects on cellular and extracellular remodeling in the infarcted hearts. It protected the heart in the early phase of infarct healing by increasing survival and proliferation of cardiomyocytes. The antiapoptotic effect of HO-1 persisted in the late phases of infarct healing. In addition, HO-1 modulated the production of extracellular matrix components and reduced perivascular fibrosis. Some of these beneficial effects of HO-1 were mediated by CO, e.g. the antiapoptotic effect. However, CO may also have adverse effects on the heart, since it increased the expression of extracellular matrix components. In isolated perfused rat hearts, HO-1 induction improved the recovery of postischemic cardiac function and abrogated reperfusion-induced ventricular fibrillation, possibly via connexin 43 (Cx43).

In the clinical study (V), HO-1 plasma levels were increased in all critically ill patients, including cardiac patients, and were associated with the degree of organ dysfunction (SOFA score) and disease severity (APACHE II and SAPS II scores). HO-1 plasma concentrations were also higher in ICU and hospital nonsurvivors than in survivors, and the maximum HO-1 concentration was an independent predictor of hospital mortality. Patients with the *HO-1* -413T/GT(L)/+99C haplotype had lower HO-1 plasma concentrations and lower level of appearance of multiple

organ dysfunction (MOD) (SOFA score > 6). However, *HO-1* polymorphisms were not associated with ICU or hospital mortality.

In conclusion, HO-1 in the experimental models played an important role in the recovery and repair of infarcted hearts. HO-1 induction potentially may protect against I/R injury and cardiac dysfunction in isolated rat hearts. Furthermore, HO-1 induction and and CO donor pretreatment enhanced cardiac regeneration after rat MI, and HO-1 may protect against pathological left ventricular remodeling. In addition, HO-1 plasma levels were significantly increased in critically ill ICU patients and correlate with the degree of organ dysfunction, disease severity, and mortality, suggesting that HO-1 may be useful as a marker of disease severity and in the assessment of outcome of critically ill patients.

1 INTRODUCTION

Myocardial infarction (MI) and heart failure are major causes of morbidity and mortality worldwide. Treatment of MI involves early restoration of blood flow to limit infarct size and preserve cardiac function. MI is followed by left ventricular remodeling, which is characterized by inflammation and subsequent formation of the fibrous scar in the infarct area to replace the damaged myocardial tissue, and cardiomyocyte apoptosis, fibrosis, and hypertrophy of the noninfarcted myocardium. These structural changes may eventually lead to the development of heart failure, despite the established pharmacological treatment of the disease. To improve outcome of MI, new strategies for protecting the myocardium against ischemia/reperfusion (I/R) injury and for promoting the healing and repair of the infarcted heart are needed.

The recent discovery of resident cardiac stem cells (CSCs) shows that the heart is a regenerating organ (Beltrami et al. 2003). However, the number of CSCs is relatively small. Therefore, activation of the resident CSCs is needed to improve cardiac repair. Angiogenic cytokines and growth factors secreted from the infarcted heart contribute to the activation of stem cells (Gnecchi et al. 2008, Srinivas et al. 2009), but the molecular mechanisms leading to the activation of CSCs are inadequately understood.

Heme oxygenase-1 (HO-1) is a stress-responsive and cytoprotective enzyme that catalyzes the degradation of heme into the biologically active reaction products biliverdin, carbon monoxide (CO) and free iron (Tenhunen 1969, Otterbein 2003). HO-1 plays a key role in maintaining cellular homeostasis (Otterbein and Choi 2000, Otterbein et al. 2003a). The cytoprotection is mediated by the antiapoptotic, anti-inflammatory, antioxidative, antiproliferative, and vasodilatory properties of HO reaction products (Stocker et al. 1987, Thorup et al. 1999, Brouard et al. 2000, Otterbein et al. 2000, Peyton et al. 2002). Interestingly, HO-1 is also known to promote angiogenesis (Dulak et al. 2008). Although HO-1 has been acknowledged as a cardioprotective protein in various cardiovascular disease models (Idriss et al. 2008, Peterson et al. 2009), the cardioprotective mechanism of HO-1 is still uncompletely understood. In addition, the role of HO-1, especially in cardiac regeneration, is not known.

HO-1 polymorphisms regulate the transcriptional activity of the *HO-1* gene in humans (Hirai et al. 2003, Ono et al. 2004, Brydun et al. 2007). *HO-1* polymorphisms have been associated with various clinical conditions, including the susceptibility to coronary artery disease and restenosis after peripheral angioplasty (Kaneda et al. 2002, Schillinger et al. 2004). However, the importance of *HO-1* polymorphisms in these conditions is controversial, since larger studies in Caucasian patients have not confirmed the association of *HO-1* polymorphisms with these conditions (Tiroch et al. 2007, Lublinghoff et al. 2009). In addition, despite the increasing number of studies on *HO-1* polymorphisms, the effect of these polymorphisms on HO-1 plasma levels is unknown. Furthermore, studies investigating HO-1 in critically ill patients, and especially in cardiac patients, are limited.

The present study aimed, first, at evaluating the role of HO-1 as a cardioprotective and prohealing enzyme in experimental rat models and at investigating the potential mechanisms mediating the beneficial effects of HO-1 in the heart. The second aim was to evaluate the role of HO-1 in critically ill patients by investigating the association of *HO-1* polymorphisms and HO-1 plasma concentrations with illness severity and mortality throughout the study population and in the subgroup of cardiac patients.

2 REVIEW OF THE LITERATURE

2.1 Heme oxygenase (HO)

HO catalyzes the first and rate-limiting step in the degradation of heme, as characterized by Tenhunen et al. (1968, 1969). They described that HO cleaves heme at the α-methene bridge, resulting in equimolar amounts of biliverdin-IXα, CO and free iron (Fig. 1) (Tenhunen et al. 1969). Oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) are required for the reaction and the central iron atom of heme is necessary for HO activity, because free porphyrin is not degraded by HO (Tenhunen et al. 1969). Subsequently, biliverdin-IXα is rapidly reduced to bilirubin-IXα by biliverdin reductase (Tenhunen et al. 1970b) and iron is sequestered in ferritin or transported out of the cells via an adenosine triphosphate (ATP)-dependent iron pump (Vile and Tyrrell 1993, Baranano et al. 2000).

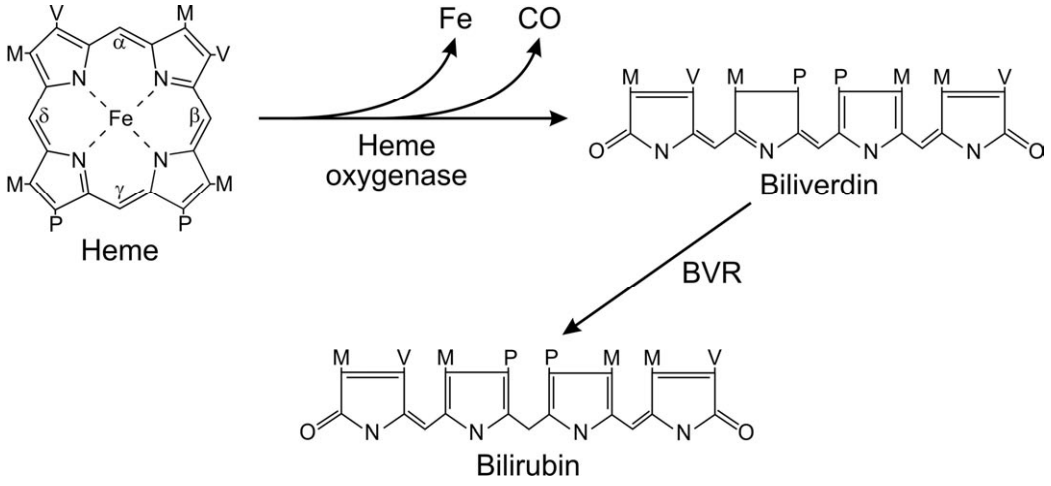

Figure 1. Heme oxygenase (HO) reaction. HO catalyzes the degradation of heme to biliverdin, carbon monoxide (CO) and free iron (Fe). Biliverdin is reduced to bilirubin by biliverdin reductase (BVR). M = methyl, V = vinyl, P = propionic acid.

Two isoforms of HO have been described. HO-1 is a 32-kDa inducible isoform, also known as heat shock protein 32 (HSP-32), and HO-2 is a 36-kDa constitutively expressed isoform (Maines et al. 1986, Keyse and Tyrrell 1989). A third isoform (HO-3) has been described in rats, but it has since become evident that it is a pseudogene derived from the HO-2 transcript (McCoubrey et al. 1997, Hayashi et al. 2004). HO-1 and HO-2 are products of two genes (Cruse and Maines 1988, Maines 1997). The *HO-1* gene is localized in chromosome 22 and the *HO-2* gene in chromosome 16 (Kutty et al. 1994). HO-1 and HO-2 have only 43% homology of amino acid sequences (Rotenberg and Maines 1990). However, they both have a highly conserved heme-catalytic domain and a similar hydrophobic region at the carboxylterminus to anchor the enzyme to the endoplasmic reticulum (Ishikawa et al. 1991, Rotenberg and Maines 1991). Both enzymes are catalytically active, and the HO enzyme activity can be inhibited using synthetic metalloporphyrins of which zinc protoporphyrin-IX (ZnPPIX) and tin protoporphyrin-IX (SnPPIX) are most commonly used (Drummond and Kappas 1981, 1982). HO-1 is a ubiquitously expressed enzyme present at low levels in most organs. Under physiological conditions, high levels of HO-1 are found only in the spleen and other tissues/cells that degrade senescent erythrocytes, such as the reticuloendothelial cells of the liver and bone marrow (Tenhunen et al. 1968, 1969). The tissue distribution of HO-2 differs from that of HO-1. High levels of HO-2 are found in the testes, brains, central nervous system, liver, kidneys, vasculature, and gut (Maines 1997).

HOs are predominantly localized in the microsomal fraction, or smooth endoplasmic reticulum, of the cells as originally described by Tenhunen et al. (1969). However, HO-1 has recently been found in other subcellular compartments as well. Kim et al. (2004) found HO-1 in the plasma membrane caveolae of endothelial cells, together with biliverdin reductase, and suggested that compartmentalization of HO-1 in the caveolae may play a role in cellular protection by modulating caveolae-mediated signaling cascades. Two years later Converso et al. (2006) showed that a fraction of HO-1 is localized in the liver mitochondria, again together with biliverdin reductase, and modulates mitochondrial heme metabolism and O_2 uptake and production of reactive oxygen species (ROS). They suggested that mitochondrial localization may explain the protective effects of HO-1 under conditions characterized by increased mitochondrial ROS production, such as I/R, sepsis, and neurodegenerative disorders (Converso et al. 2006). Lin and colleagues showed that exposure to hypoxia or hemin resulted in nuclear localization of a truncated form of HO-1 lacking the C-terminus of the protein (Lin et al. 2007). They also demonstrated that despite the decreased enzyme activity of this truncated nuclear HO-1, it was equally cytoprotective, presumably by activating transcription factors that are involved in the oxidative stress response, including activator protein 1 (AP-1) (Lin et al. 2007). In addition, Lin et al. (2008) showed that the ubiquitin-proteasome system mediates HO-1 degradation via the endoplasmic reticulum-associated degradation pathway.

2.2 Induction and regulation of HO-1

2.2.1 HO-1-inducing factors

HO-1 expression and activity are highly induced by numerous factors. The induction of HO enzyme activity by its substrate heme was reported by Tenhunen et al. (1970a) soon after they had characterized the enzyme. In the late 1980s, Shibahara et al. (1987) showed that HO-1 expression was increased by heat shock in rats and suggested that HO-1 is a heat shock protein. This was later confirmed by different groups, showing that the 32-kDa stress response protein, induced by numerous factors such as heavy metals and ultraviolet A (UVA) radiation, is HO-1 (Keyse and Tyrrell 1987, 1989, Taketani et al. 1989). However, although HO-1 is up-regulated in response to heat shock in rats (Taketani et al. 1988, Raju and Maines 1994), it is not induced by hyperthermia in humans (Yoshida et al. 1988, Shibahara et al. 1989, Taketani et al. 1989).

An ever-growing number of HO-1-inducing stimuli has been characterized since these studies in the 1980s, and the role of HO-1 as a stress response protein has become evident (Otterbein and Choi 2000). Some of the HO-1-inducing stimuli are listed in Table 1. A common feature for many of the HO-1-inducing factors and conditions is that they cause oxidative stress by increasing the production of ROS or decreasing intracellular glutathione levels (Ryter et al. 2006). It is also worth noting that several metalloporphyrins (e.g. ZnPPIX and SnPPIX) can paradoxically induce HO-1 expression, although they also inhibit HO enzyme activity (Sardana and Kappas 1987). Due to the high inducibility of HO-1 along with its cytoprotective effects, Otterbein and colleagues (2003a) have suggested that HO-1 may function as a 'therapeutic funnel' mediating the beneficial effects of other molecules, such as interleukin (IL)-10, prostaglandin J2 and HSPs (Lee and Chau 2002, Redaelli et al. 2002, Lee et al. 2003, Otterbein et al. 2003a).

Table 1. Inducers of HO-1 expression or enzyme activity.

Stimulus	Reference
Heme	Alam et al. 1989
Heavy metals (cadmium, cobalt)	Caltabiano et al. 1986, Taketani et al. 1988, 1989
Hypoxia	Borger and Essig 1998, Panchenko et al. 2000
Hyperoxia	Lee et al. 2000
Ischemia/reperfusion	Raju and Maines 1996
Reactive oxygen species	Keyse et al. 1990, Keyse and Emslie 1992, Ohlmann et al. 2003
Shear stress	Wagner et al. 1997, De Keulenaer et al. 1998
Lipopolysaccharide	Rizzardini et al. 1994, Camhi et al. 1995
Nitric oxide, peroxynitrite	Durante et al. 1997, Hartsfield et al. 1997, Foresti et al. 1999
Cytokines (IL-1α, IL-10, IL-11, IL-6, TNF-α, TGF-β)	Fukuda and Sassa 1993, Terry et al. 1998, 1999, Lee and Chau 2002, Ning et al. 2002
Growth factors (VEGF, PDGF)	Durante et al. 1999, Bussolati et al. 2004, Bussolati and Mason 2006
Drugs (aspirin, statins)	Grosser et al. 2003, 2004, Lee et al. 2004
Prostaglandins	Lee et al. 2003, Alvarez-Maqueda et al. 2004
ACTH	Pomeraniec et al. 2004
Angiotensin II	Ishizaka and Griendling 1997, Ishizaka et al. 2000
Antioxidants (curcumin, caffeic acid, resveratrol)	Motterlini et al. 2000b, Scapagnini et al. 2002, Chen et al. 2005

Abbreviations: ACTH = adrenocorticotrophic hormone, HO-1 = heme oxygenase-1, IL = interleukin, PDGF = platelet-derived growth factor, TGF-β = transforming growth factor-β, TNF-α = tumor necrosis factor α, VEGF = vascular endothelial growth factor.

2.2.2 Regulation of HO-1 expression

The innumerable amount of HO-1 inducers points to the presence of several response elements in the promoter of the *HO-1* gene and numerous interactions between the components of different signaling pathways. Induction of HO-1 by various factors involves activation of different protein phosphorylation cascades, including mitogen-activated protein kinases (MAPKs), tyrosine kinases, phosphatidylinositol 3-kinase (PI3K), and protein kinases A, C, and G (Ryter et al. 2006). MAPK cascades are activated by cellular stress and they also regulate cell proliferation and differentiation (Cobb 1999). The MAPK system is composed of three signaling pathways: the extracellular-regulated kinases (ERK pathway), the c-Jun N-terminal kinases (JNK pathway), and the p38 kinases (p38 pathway). The MAPKs are major mediators of the HO-1 stress response and HO-1 inducers may activate one or more of these MAPK pathways (Ryter et al. 2006, Alam and Cook 2007). However, the p38 pathway is the major MAPK cascade activating HO-1 in response to various stresses. Sodium arsenite activates all three MAPK pathways in hepatoma cells and induces HO-1 (Elbirt et al. 1998). Induction of HO-1 by nitric oxide (NO) involves both the p38 MAPK and ERK pathways in HeLa cells (Chen and Maines 2000), whereas hypoxia induces HO-1 in rat cardiomyocytes via the p38 MAPK pathway (Kacimi et al. 2000).

The *HO-1* gene contains three major regulatory regions (Fig. 2); a proximal promoter region at 0.3 kb upstream from the transcription initiation site and two distal enhancer regions at 4 and 10 kb upstream from the transcription initiation site (Alam et al. 1994, 1995, Alam and Cook 2007). The dominant regulatory element in the distal enhancers is the stress-responsive

element (StRE), which is similar to the Maf response element (MARE) and the antioxidant response element (ARE), and contains the heme response element and the AP-1 binding site (Ryter et al. 2006). Several transcription factors bind to the StRE as hetero- or homodimers, including AP-1 factors (Jun and Fos), Maf proteins, and the cap'n'collar/basic-leucine zipper transcription factors Nrf2 (nuclear factor-erythroid 2-related factor 2) and Bach1 (Alam and Den 1992, Alam et al. 1999, Sun et al. 2002, 2004). The AP-1 factors mediate the activation of the *HO-1* gene in response to hyperoxia and lipopolysaccharide (LPS) (Camhi et al. 1995, Lee et al. 2000). However, the major transcriptional regulator of the *HO-1* gene is the ARE-binding transcription factor Nrf2 (Ryter et al. 2006). Nrf2 is a positive regulator of antioxidant, cytoprotective, and anti-inflammatory genes containing the ARE sequence (Motohashi and Yamamoto 2004, Levonen et al. 2007, Jyrkkänen et al. 2008). Both Nrf2 and Bach1 form heterodimers with small Maf proteins. Nrf2 increases *HO-1* transcription, while Bach1 competes with Nrf2 and represses *HO-1* transcription (Alam et al. 1999, Sun et al. 2002, 2004). Heme binds with Bach1, inhibits its DNA-binding activity, and promotes its nuclear export, thus enhancing HO-1 expression (Sun et al. 2002, Suzuki et al. 2004). In addition, a cytoplasmic Kelch-like ECH-associated protein 1 (Keap1) binds Nrf2 under basal conditions, prevents its translocation to the nucleus, and facilitates its degradation, thus reducing HO-1 expression (Itoh et al. 1999, 2003). When cells are exposed to oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus, and binds to its target genes (Dinkova-Kostova et al. 2002).

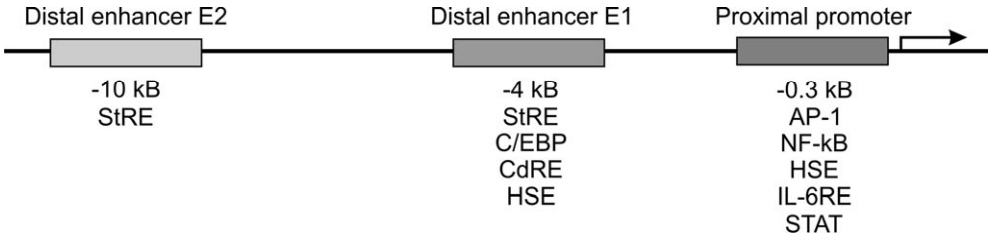

Figure 2. Simplified scheme of the regulatory elements in the *heme oxygenase-1* gene promoter. The arrow marks the transcription initiation site. StRE = stress-responsive element, C/EBP = CAAT/enhancer-binding protein site, CdRE = cadmium-responsive element, HSE = heat shock element, AP-1 = activator protein-1, NF-κB = nuclear factor-κB, IL-6RE = interleukin-6-responsive element, STAT = signal transducer and activator of transcription.

In addition to StRE, many other regulatory elements have been found in the HO-1 promoter, including the cytidine-adenosine-adenosine-thymidine (CAAT)/enhancer-binding protein (C/EBP) site and the cadmium-responsive element (CdRE) (Ryter et al. 2006). Binding sites for nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (Stat3) and hypoxia-inducible factor 1α (HIF-1α) have also been identified, as well as IL-6, heat shock and metalloporphyrin-responsive elements (Shibahara et al. 1989, Lavrovsky et al. 1994, Lee et al. 1997, Deramaudt et al. 1999, Yang et al. 2001, Ryter et al. 2006). Although Nrf2 is the major regulator of the *HO-1* gene, the presence of the above-mentioned and several other regulatory elements in the *HO-1* promoter corroborate the role of HO-1 as a stress response protein.

The transcriptional activity of the human *HO-1* gene is also affected by the following *HO-1* promoter polymorphisms: the GT_n repeat length polymorphism and -413A/T single-nucleotide polymorphism (SNP). The short GT_n repeat length allele and -413A allele enhance transcriptional activity of the *HO-1* gene compared with the long GT_n and -413T alleles (Hirai et al. 2003, Ono et al. 2004, Brydun et al. 2007). The *HO-1* promoter polymorphisms may be of functional importance in clinical conditions, since they have been associated e.g. with susceptibility to coronary artery disease and restenosis after peripheral angioplasty in some studies (Kaneda et al. 2002, Schillinger et al. 2004). However, in some studies no association was found between *HO-1* polymorphisms and the disease studied (Tiroch et al. 2007, Turpeinen

et al. 2007). Table 2 summarizes the studies investigating *HO-1* polymorphisms in different diseases.

Table 2. Major studies investigating *HO-1* polymorphisms in different diseases.

Disease	Polymorphism	N	Association with disease	Reference
Cardiovascular disease				
CAD	GTn, -413A/T	3219	No	Lublinghoff et al. 2009
CAD in diabetic patients	GTn	986	Yes	Chen et al. 2008
Coronary atherosclerosis	GTn	110	Yes	Brydun et al. 2007
CAD in type II diabetic patients	GTn	796	Yes	Chen et al. 2002
CAD in patients with risk factors	GTn	577	Yes	Kaneda et al. 2002
CAD	GTn	649	No	Endler et al. 2004
CAD	-413A/T	2569	Yes	Ono et al. 2004
Cardiovascular adverse effects in peripheral artery disease patients	GTn	472	Yes	Dick et al. 2005
Hypertension in women	-413A/T	1998	Yes	Ono et al. 2003
Restenosis after coronary stenting	GTn	1807	No	Tiroch et al. 2007
Restenosis after coronary stenting	GTn, +99G/C	199	GTn Yes +99G/C No	Gulesserian et al. 2005
Restenosis after coronary stenting	GTn	323	Yes	Chen et al. 2004
Restenosis after balloon angioplasty	GTn	381	Yes	Schillinger et al. 2004
Heart failure and cardiac transplantation outcome	GTn	592	No	Holweg et al. 2005
Cardiac allograft vasculopathy	GTn	152	No	Ullrich et al. 2005
Risk of recurrent venous thromboembolism	GTn	860	Yes	Mustafa et al. 2008
Renal disease				
Renal transplantation outcome	GTn, -413A/T	1125	No	Turpeinen et al. 2007
Renal transplantation outcome	GTn	1414	No	Courtney et al. 2007
Renal allograft function	GTn	101	Yes	Exner et al. 2004
Renal allograft outcome	GTn	771	Yes	Baan et al. 2004
Pulmonary disease				
ARDS	GTn, S-TAG haplotype	1451	Yes	Sheu et al. 2009
Chronic pulmonary emphysema	GTn	201	Yes	Yamada et al. 2000
Susceptibility to pneumonia	GTn	400	Yes	Yasuda et al. 2006
Lung function decline	GTn	749	Yes	Guenegou et al. 2006
Other diseases				
Gastric adenocarcinoma	GTn	433	Yes	Lo et al. 2007
Gastric cancer	GTn	317	Yes	Sawa et al. 2008
Liver transplantation outcome	GTn, -413A/T	308	GTn No -413A/T Yes	Buis et al. 2008
Idiopathic recurrent miscarriage	GTn	291	Yes	Denschlag et al. 2004
Type II diabetes	GTn, -413A/T, Haplotypes	3089	GTn Yes -413A/T No Haplotypes Yes	Song et al. 2009a
Rheumatoid arthritis	GTn, -413A/T, Haplotypes	1582	GTn Yes -413A/T No Haplotypes Yes	Rueda et al. 2007

Abbreviations: ARDS = acute respiratory distress syndrome, CAD = coronary artery disease, HO-1 = heme oxygenase-1. S-TAG haplotype includes GTn S-allele, -413 T-allele, rs2071748 A-allele and rs5755720 G-allele.

2.3 Protective role of HO-1 and its reaction products

It was originally assumed that HO functions only in the basic metabolism of heme, and HO reaction products were considered as waste products with potential toxic effects. Indeed, high levels of inhaled CO are known to impair O_2 transport and cause tissue hypoxia, high levels of bilirubin can cause neonatal jaundice and neurologic damage, and iron is a pro-oxidant. However, during the last decade HO-1 has established its role as a cytoprotective enzyme in various tissues and conditions, including the cardiovascular system (Otterbein et al. 2003a, Peterson et al. 2009). The cytoprotective mechanism of HO-1 is still unclear, but the beneficial effects of HO-1 are presumably mediated by the degradation of pro-oxidative heme and production of biologically active HO reaction products. Biliverdin and bilirubin are powerful antioxidants (Stocker et al. 1987). CO mediates the antiapoptotic, anti-inflammatory, antiproliferative and vasodilatory properties of HO-1 (Thorup et al. 1999, Brouard et al. 2000, Otterbein et al. 2000, Peyton et al. 2002), and iron induces the synthesis of ferritin, which is also a cytoprotective molecule and sequesters free iron (Vile and Tyrrell 1993). The beneficial effects of CO and biliverdin/bilirubin are shown in Fig. 3.

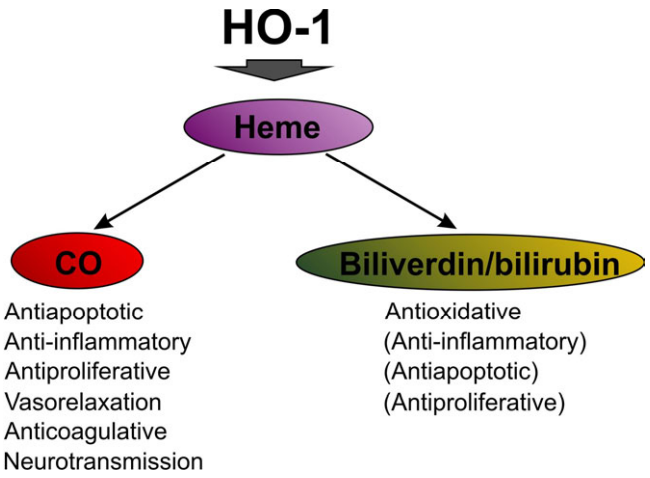

Figure 3. Protective effects of the heme oxygenase (HO) reaction products carbon monoxide (CO) and biliverdin/bilirubin.

2.3.1 Antioxidant effect

The antioxidant effect of HO-1 is mediated by biliverdin and bilirubin. In 1987, Stocker et al. showed that bilirubin scavenges peroxyl radicals, and the antioxidant activity of bilirubin increases in hypoxic conditions. Biliverdin and bilirubin also scavenge other ROS, including superoxide, hydroxides, hypochlorous acid, and singlet oxygen (Nakamura et al. 1987, Stocker and Peterhans 1989, Stocker 2004). In addition, biliverdin and bilirubin scavenge reactive nitrogen species such as peroxynitrite (Kaur et al. 2003, Mancuso et al. 2003). Bilirubin can protect cells from a 10 000-fold excess of hydrogen peroxide (H_2O_2) (Baranano et al. 2002). The powerful scavenging of reactive oxygen and nitrogen species by bilirubin was explained by an amplification cycle, whereby bilirubin is oxidized to biliverdin and recycled back to bilirubin by biliverdin reductase (Baranano et al. 2002). However, Maghzal et al. (2009) showed that the bilirubin-biliverdin redox amplification cycle plays only a limited role in cellular anti-oxidant defense. Similarly, Jansen et al. demonstrated recently (2010) that bilirubin is more efficient antioxidant than biliverdin, and conversion of biliverdin to bilirubin contributes to

cytoprotection by HO-1. Bilirubin generated by HO-1 protects against oxidative stress in vascular smooth muscle cells (VSMCs) (Clark et al. 2000a). In addition, bilirubin and biliverdin protect against I/R injury in kidneys and liver (Fondevila et al. 2004, Adin et al. 2005).

2.3.2 Antiapoptotic effect

The effect of HO-1 is antiapoptotic in most cells and conditions, and the antiapoptotic effect is predominantly mediated by CO. HO-1 prevented tumor necrosis factor α (TNF-α)-induced apoptosis in fibroblasts, presumably via CO (Petrache et al. 2000). In addition, CO inhibits TNF-α-induced apoptosis of endothelial cells via the p38 MAPK pathway (Brouard et al. 2000). Brouard et al. (2002) also showed that inhibition of TNF-α-mediated apoptosis by HO-1/CO requires activation of transcription factor NF-κB. However, different signaling pathways may be involved in the antiapoptotic effect of CO in different cells and conditions. In VSMCs, the antiapoptotic effect of CO was mediated partly by guanosine 3'5'-cyclic monophosphate (cGMP) (Liu et al. 2002a). In some circumstances, HO-1 and CO may also increase apoptosis, since CO increases endothelial cell apoptosis by increasing NO (Thom et al. 2000). In addition, Liu et al. (2002b) showed that overexpression of HO-1 in rat aortic smooth muscle cells (SMCs) stimulates apoptosis, and the proapoptotic effect of HO-1 involves bilirubin/biliverdin. However, in the majority of experimental models HO-1 and CO have protected against apoptosis. HO-1 and CO prevented inflammation-related apoptotic liver damage in mice (Sass et al. 2003) and inhibited apoptosis in transplanted lungs and hearts (Song et al. 2003, Akamatsu et al. 2004). The antiapoptotic effect of HO-1 may also be mediated by HO reaction products other than CO. Ferris et al. (1999) showed that HO-1 inhibits apoptosis by augmenting iron efflux from the cells. In addition, HO-1 overexpression inhibits Fas-mediated apoptosis in Jurkat T cells by an iron-dependent mechanism (Choi et al. 2004). The third HO reaction product, bilirubin, may protect against peroxynitrite-induced apoptosis (Foresti et al. 1999). Bilirubin also inhibited bile acid-induced apoptosis of rat hepatocytes by its antioxidative action (Granato et al. 2003). However, bilirubin is toxic to brains and increases apoptosis of neurons *in vitro* (Silva et al. 2002).

2.3.3 Anti-inflammatory effect

The anti-inflammatory action of HO-1 was demonstrated in 1996 by Willis et al. The anti-inflammatory role of HO-1 is supported by the chronic inflammation of HO-1 null mice and the severe inflammatory syndrome of the only person diagnosed to date with HO-1 deficiency (Poss and Tonegawa 1997a, 1997b, Yachie et al. 1999, Kawashima et al. 2002). The anti-inflammatory effect of HO-1 is predominantly mediated by CO. HO-1/CO suppress the production of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and monocyte chemotactic protein 1 (MCP-1) and enhance the anti-inflammatory response by increasing the expression of the anti-inflammatory cytokine IL-10 (Otterbein et al. 2000, Morse et al. 2003). Furthermore, IL-10 increases the expression of HO-1, and the anti-inflammatory effect of IL-10 is dependent on HO-1 (Lee and Chau 2002). These findings point to a positive feedback loop for amplifying the anti-inflammatory effect of CO. Likewise, in chronic murine cholitis the anti-inflammatory effect of CO was dependent on HO-1 induction (Hegazi et al. 2005). Otterbein et al. (2000) showed that the anti-inflammatory effect of CO is mediated by the MAPK pathway, but does not involve cGMP or NO. In addition, HO-1 decreases production of proinflammatory granulocyte macrophage colony-stimulating factor (GM-CSF) via the NF-κB pathway (Sarady et al. 2002). The anti-inflammatory effect of HO-1 may partly result from the down-regulation of adhesion molecules, such as E- and P-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) (Hayashi et al. 1999, Rucker et al. 2001, Soares et al. 2004, Song et al. 2009b). Down-regulation of adhesion molecules decreases infiltration of leukocytes

in the injured tissue and thereby attenuates inflammation. In addition, the inhibition of platelet aggregation and subsequent thrombosis may contribute to the anti-inflammatory effect of HO-1/CO (Brune and Ullrich 1987).

2.3.4 Antiproliferative effect

HO-1 inhibits proliferation of several cell types, such as SMCs, T cells, and fibroblasts (Peyton et al. 2002, Song et al. 2002, Song et al. 2004, Zhou et al. 2005, Liu et al. 2006). The antiproliferative effect of HO-1 is predominantly mediated by CO, as shown in VSMCs by Morita et al. (1995, 1997). Although CO inhibits proliferation of several cell types, the signaling pathways involved in the antiproliferative effect of CO differ considerably in a cell type-specific manner. In VSMCs the antiproliferative effect of CO involves production of cGMP, activation of the p38 MAPK pathway, expression of the cell cycle inhibitor $p21^{Cip1}$, and inhibition of the cell cycle-specific transcription factor E2F-1 (Morita et al. 1997, Otterbein et al. 2003b). In airway SMCs the antiproliferative effect of CO is mediated by down-regulation of the ERK MAPK pathway (Song et al. 2002), and in T cells the antiproliferative effect of CO involves increased expression of $p21^{Cip1}$ and decreased caspase-8 activity and is independent of the cGMP and MAPK pathways (Song et al. 2004). Biliverdin and bilirubin may also regulate cell proliferation, since Ollinger et al. (2005) showed that bilirubin decreases SMC proliferation by inhibiting cyclins A, D1, and E, and cyclin-dependent kinase 2 (cdk2) via the p38 MAPK pathway, and decreases neointimal formation after balloon injury. In addition, it should be noted that HO-1 and CO increase proliferation of some cell types, such as endothelial cells and regulatory T cells (Li Volti et al. 2002, Brusko et al. 2005, Lee et al. 2007). These findings highlight the importance of HO-1 and CO, e.g. in vascular remodeling.

2.3.5 Vasoactive effects

HO-1 regulates vascular tone by a CO-dependent mechanism. Similar to NO, CO activates soluble guanylate cyclase (sGC) and increases cGMP levels, leading to vasodilation (Morita et al. 1995, Sammut et al. 1998, Duckers et al. 2001). The vasodilatory effect of CO was reported in 1978 by Sylvester and McGowan, and in 1988 Lin and McGrath showed in rat aortas that the effect of CO was not endothelium-dependent. Furchgott and Jothianandan (1991) compared the vasoactive effects of NO and CO in isolated rabbit aorta and found that similar to NO, CO caused vasodilation via production of cGMP, although CO was 1000-fold less potent than NO as a vasorelaxant. To confirm the hypothesis that cGMP mediates CO-induced vasorelaxation, Hussain et al. (1997) showed in rabbit aortic rings that CO-dependent vasodilation is abolished by the specific sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ). CO may also cause vasorelaxation via mechanisms other than by increasing cGMP levels, since CO activates calcium-dependent potassium (K_{Ca}) channels (Wang et al. 1997, Jaggar et al. 2002). Wang et al. (1997) showed that the vasodilatory effect of CO was mediated partially via cGMP and partially via large-conductance K_{Ca} channels. SMC-derived CO may also have paracrine effects on endothelial cells and regulate vascular tone by modulating the expression of endothelin 1 (ET-1) and platelet-derived growth factor β (PDGF-β) in endothelial cells (Morita et al. 1995). In some circumstances, the vasoregulatory effect of CO may also involve regulation of NO, since Foresti et al. (2004) showed that the vasodilatory effect of CO-releasing molecule 3 (CORM-3) was dependent on endothelium-derived NO. In some models, CO may also have a vasoconstrictive effect. Johnson et al. (2002, 2003) showed that CO may have a competing vasoconstrictive effect by inhibiting the formation of NO in endothelium. Furthermore, high concentrations of CO inhibit NO production and endothelial NO synthase (eNOS) activity in isolated renal resistance vessels (Thorup et al. 1999). Imai et al. (2001) showed that HO-1 overexpression in VSMCs inhibits formation of NO, which leads to elevated blood pressure.

2.3.6 Anticoagulative effect

HO-1 inhibits platelet activation and aggregation via a CO-dependent mechanism. Inhibition of platelet aggregation by CO was reported in 1982 by Mansouri and Perry. Brune and Ullrich showed a few years later (1987) that CO prevents platelet aggregation by activating sGC and increasing cGMP levels. In contrast, Chlopicki et al. showed recently (2006) that CO released by CORM-3 inhibited platelet aggragation by a mechanism independent of sGC. The suppression of vascular thrombosis has been shown in several studies. HO-1 induction by hemin delayed microvascular thrombus formation in mice *in vivo*, and bilirubin prevented thrombus formation as efficiently as hemin treatment, suggesting equally important antithrombotic roles for bilirubin and CO (Lindenblatt et al. 2004). Recently, Johns et al. (2009) showed that HO-1 induction by cobalt protoporphyrin IX (CoPPIX) inhibited thrombus formation in cremaster arterioles. Furthermore, True et al. (2007) showed that HO-1 null mice have accelerated thrombosis, due to increased endothelial cell apoptosis, platelet activation, and elevated tissue factor and von Willebrand factor (vWF) levels. They also demonstrated that both inhaled CO and biliverdin administration rescued the prothrombotic phenotype of HO-1 null mice (True et al. 2007). In addition, CO down-regulates the expression of prothrombotic plasminogen activator inhibitor type 1 (PAI-1) in macrophages and enhances fibrinolysis (Fujita et al. 2001). Likewise, Chen et al. (2006) showed that HO-1 overexpression and CO inhalation decreased PAI-1 levels and induced early thrombolysis after vascular injury in hypercholesterolemic mice. Moreover, Matsumoto et al. (2006) showed that both CO and bilirubin down-regulate PAI-1 expression when administered to HO-1-deficient cells.

2.3.7 Proangiogenic effect

A growing body of evidence shows that HO-1 plays an important role in angiogenesis. Angiogenesis is defined as formation of new capillaries from pre-existing capillaries by increased migration and proliferation of endothelial cells (sprouting). The increased proliferation of endothelial cells in response to HO-1 overexpression was demonstrated in 1998 by Deramaudt et al. Later studies showed that the proangiogenic effect of HO-1 in endothelial cells is mediated by CO (Jozkowicz et al. 2003, Li Volti et al. 2005). CO also inhibits endothelial cell apoptosis (Soares et al. 2002). The proangiogenic mechanism of HO-1 involves induction of angiogenic growth factors and cytokines. Overexpression of HO-1 increases vascular endothelial growth factor (VEGF) expression in endothelial cells and VSMCs (Dulak et al. 2002, Jozkowicz et al. 2003). HO-1 also promotes neovascularization in rat and mouse hindlimb ischemia models by inducing VEGF and stromal cell-derived factor 1 (SDF-1) (Suzuki et al. 2003, Tongers et al. 2008). In addition, several factors, including prostaglandin J and H_2O_2, induce VEGF synthesis via an HO-1-mediated mechanism (Jozkowicz et al. 2002, Cisowski et al. 2005). Conversely, HO-1 and CO may also be involved in the downstream response of cells to VEGF and SDF-1 stimulation, since VEGF and SDF-1 induce HO-1 expression (Bussolati et al. 2004, Deshane et al. 2007). Furthermore, inhibition of HO-1 attenuates VEGF-induced endothelial cell proliferation and tube formation (Bussolati et al. 2004). However, the effect of HO-1 on angiogenesis may differ, depending on conditions. Bussolati et al. (2004) showed that HO-1 inhibits inflammation-induced angiogenesis by preventing leukocyte infiltration, but enhances VEGF-induced noninflammatory angiogenesis. Nevertheless, these findings suggest a positive feedback loop between HO-1 and VEGF in promoting angiogenesis. Interestingly, Bellner et al. showed recently (2009) that deletion of the constitutive isoform HO-2 caused endothelial cell activation and promoted massive inflammation-driven angiogenesis, and this effect was reversed by addition of biliverdin to the HO-2 (-/-) endothelial cells. This supports the above-mentioned hypothesis that the HO system suppresses inflammation-induced angiogenesis (Bellner et al. 2009).

HO-1 and CO may potentially regulate angiogenesis via other mechanisms as well. HIF-1 is a transcription factor that regulates the expression of several angiogenic factors and induces angiogenesis in response to hypoxia (Kelly et al. 2003). HIF-1 is known to up-regulate HO-1 expression, whereas CO up-regulates and stabilizes HIF-1α (Chin et al. 2007, Faleo et al. 2008). These findings suggest another positive feedback loop that could enhance the angiogenic effect of HO-1 and CO. HO-1 may also regulate the proangiogenic cytokine IL-8 (Pae et al. 2005). In addition to the up-regulation of proangiogenic factors, HO-1 overexpression down-regulates antiangiogenic factors, such as VEGF receptor 1 (VEGF-R1) and soluble endoglin (Cudmore et al. 2007). Interestingly, Taha et al. showed recently (2010) that *HO-1* GT_n microsatellite polymorphism modulates the function of endothelial cells. GT_n polymorphism affected HO-1 expression and cells carrying the S allele survived better under oxidative stress, produced lower levels of proinflammatory mediators, and proliferated more efficiently in response to VEGF, although *HO-1* polymorphisms did not influence migration and sprouting of capillaries (Taha et al. 2010). Thus, *HO-1* polymorphisms may modulate the angiogenic potential of endothelial cells in different diseases.

2.3.8 Loss of protection in HO-1 deficiency

The only known case of human HO-1 deficiency and studies on HO-1 knockout mice highlight the crucial cytoprotective role of HO-1. The HO-1 knockout mice exhibited anemia and disturbed iron metabolism characterized by low serum iron levels and increased accumulation of iron in tissues, especially in kidneys and liver (Poss and Tonegawa 1997b). The HO-1 knockout mice also had progressive chronic inflammation, splenomegaly, lymphadenopathy, leukocytosis, vascular injury, glomerulonephritis, and premature mortality (Poss and Tonegawa 1997b). Furthermore, fibroblasts derived from HO-1 null mice are more susceptible to cytotoxicity induced by different pro-oxidant stimuli (Poss and Tonegawa 1997a). In various disease models, HO-1 deficiency predisposes to right MI after chronic hypoxia and to arterial thrombosis after vascular injury (Yet et al. 1999, True et al. 2007). HO-1 deficiency also worsens myocardial I/R injury, especially in concert with diabetes (Liu et al. 2005).

Similar findings have been reported in human HO-1 deficiency (Yachie et al. 1999, Ohta et al. 2000, Kawashima et al. 2002). The symptoms and findings in the HO-1-deficient patient are listed in Table 3. The main findings in the HO-1-deficient boy were severe systemic vascular endothelial cell injury, severe hemolytic anemia with paradoxically high serum haptoglobin and low bilirubin levels, and reticuloendothelial dysfunction (Yachie et al. 1999). The patient also had tissue iron and amyloid deposition, progressive renal tubular injury, and abnormal coagulation/fibrinolysis (Yachie et al. 1999, Ohta et al. 2000, Kawashima et al. 2002). In comparison to the HO-1 null mice, the HO-1-deficient boy was more severely affected by oxidative stress, and the cells of the patient were extremely sensitive to hemin-induced cell injury (Poss and Tonegawa 1997b, Yachie et al. 1999). The high serum levels of pro-oxidative heme likely contributed to the severe vascular endothelial cell injury. The severe injuries may also have partially resulted from exposure of the patient to infectious agents and other stress factors, starting soon after birth (Ohta et al. 2000). In contrast to the HO-1 null mice having splenomegaly, the HO-1 deficient patient had no spleen. The absence of spleen likely contributed to the endothelial cell injury and tubulus injury, due to absence of the splenic filtering function (Ohta et al. 2000). The patient died early (at the age of 6 years) of an intracranial hemorrhage and progressed disease (Kawashima et al. 2002). This case of human HO-1 deficiency corroborates the function of HO-1 as a ubiquitous stress response protein.

Table 3. Symptoms and clinical findings in the heme oxygenase-1-deficient patient.

Symptom/Finding	Symptom/Finding
Recurrent fever	Leukocytosis
Erythematous rash	Thrombocytosis
Growth retardation	Abnormal coagulation/fibrinolysis, DIC
Hepatomegaly	Vascular endothelial injury
Asplenia	High thrombomodulin and von Willebrand factor
Cervical lymphadenopathy	Reticuloendothelial dysfunction
Intravascular hemolytic anemia	Hematuria and proteinuria
High serum haptoglobin	Renal tubular injury
High lactate dehydrogenase	High urinary N-acetyl-B-D-glucosaminidase
Low bilirubin	High urinary B2-microglobulin
High ferritin	Mesangioproliferative glomerulonephritis
Coombs test negative	Iron deposition in liver and kidneys
High serum heme	Amyloid deposits in liver and adrenal glands
Undetectable hemopexin	Hyperlipidemia
High aspartate aminotransferase	Atherosclerotic changes

Abbreviations: DIC = disseminated intravascular coagulopathy.

2.4 HO-1 and its reaction products in cardiovascular diseases

Increasing numbers of studies show that HO-1 and its reaction products protect the heart and vasculature in pathological conditions. Increased expression of HO-1 has been found in the heart and vasculature *in vivo* and in cardiomyocytes, endothelial cells, and VSMCs *in vitro* in response to various stimuli, such as hyperthermia, I/R, hypoxia, cytokines, hemin, NO and angiotensin II (Raju and Maines 1994, Maulik et al. 1996, Motterlini et al. 1996, Durante et al. 1997, Lee et al. 1997, Pellacani et al. 1998, Terry et al. 1998, Clark et al. 2000b, Hangaishi et al. 2000, Ishizaka et al. 2000, Motterlini et al. 2000a). Overexpression or pharmacological induction of HO-1, or administration of HO reaction products, provides protection e.g. in MI and heart failure, atherosclerosis and vascular injury, and hypertension in experimental rat, mouse, and swine models. The beneficial effects of HO-1 in various disease models are summarized in Fig. 4.

2.4.1 HO-1 in myocardial infarction and heart failure

HO-1 and its reaction products have both short-term and long-term protective effects on myocardial I/R injury and MI. The crucial stress-responsive role of HO-1 was demonstrated by increased right ventricular dilatation, right ventricular infarction, and mural thrombi of HO-1 null mice after chronic hypoxia (Yet et al. 1999). Conversely, cardiac-specific overexpression of HO-1 improves postischemic cardiac function, decreases infarct size, and reduces cardiac apoptosis, inflammatory cell infiltration, and oxidative damage in I/R mouse hearts (Yet et al. 2001, Vulapalli et al. 2002). Likewise, cardiac gene transfer of HO-1 8 weeks before I/R decreased infarct size, inflammation, and expression of proapoptotic and proinflammatory proteins in rat hearts (Melo et al. 2002). In addition, HO-1 induction by hemin decreased infarct size and improved postischemic cardiac function in I/R rat hearts, and the protective effect was mediated by bilirubin (Clark et al. 2000b). Bilirubin also protects against reoxygenation damage in rat cardiomyocytes *in vitro* (Foresti et al. 2001).

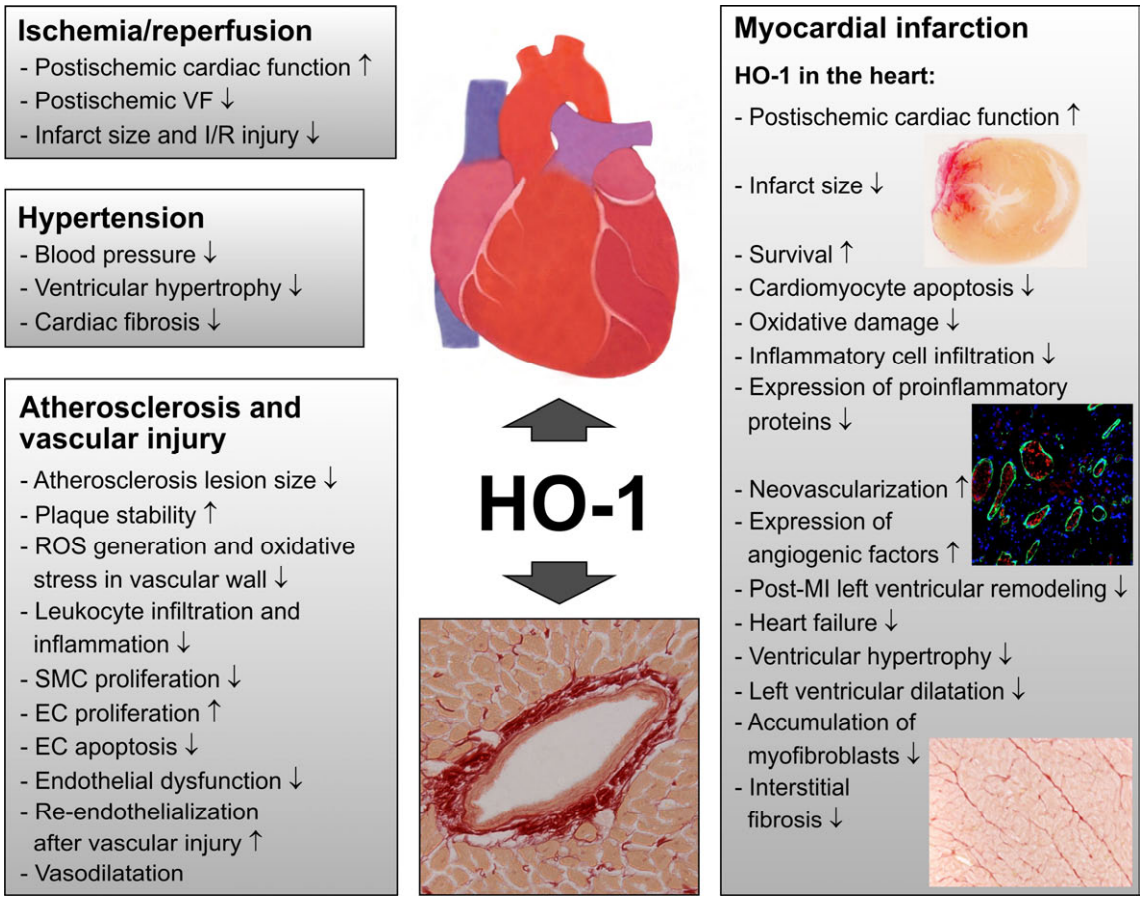

Figure 4. Beneficial effects of HO-1 in the heart and vasculature in different disease models. Abbreviations: EC = endothelial cell, HO-1 = heme oxygenase-1, I/R = ischemia/reperfusion, ROS = reactive oxygen species, SMC = smooth muscle cell, VF = ventricular fibrillation.

CO exerts protective effects in the setting of cardiac I/R as well. Bak et al. (2005) showed in isolated I/R rat hearts that CO exposure via perfusion buffer decreases infarct size, improves postischemic cardiac function, and decreases the incidence of I/R-induced ventricular fibrillation (VF). The same group demonstrated that pretreatment of rat hearts with the CO donor CORM-3 similarly protected the heart during I/R, and the protective effect involved the regulation of cardiac Na^+, K^+, and Ca^{2+} levels (Varadi et al. 2007). Guo et al. (2004) showed that administration of CORM-3 at the time of reperfusion decreased infarct size in mouse hearts *in vivo* after 30 min of focal ischemia and 24 h of reperfusion. In addition, administration of CORM-3 24–72 h before I/R induced delayed protection against MI similar to the late phase of ischemic preconditioning (Stein et al. 2005). Furthermore, rapid release of CO by CORM-3 exerts a positive inotropic effect on isolated perfused rat hearts, and this effect involved cGMP and Na^+/H^+ exchange (Musameh et al. 2006). However, slow release of CO by CORM-A1 did not affect myocardial contractility, but caused vasodilatation (Musameh et al. 2006). Based on these studies, brief exposure to CO protects the heart against I/R injury. However, Meyer et al. showed recently (2010) that prolonged exposure to 30–100 parts per million (ppm) of CO by inhaled air worsens myocardial I/R injury, increases the severity of postischemic ventricular arrhythmias, impairs postischemic cardiac function, and increases infarct size in rats.

The cytoprotective and prongiogenic effects of HO-1 point to a potential role for HO-1 in cardiac cell therapy and cardiac regeneration after MI. Transplantation of mesenchymal stem cells (MSCs) in the infarcted rat hearts increased HO-1 expression in the transplanted MSCs and in the cardiomyocytes of the recipient heart, decreased infarct size, and improved cardiac function

(Zhang et al. 2005). HO-1 also improved survival of MSCs in the infarcted rat and mouse hearts during the first week after MI (Tang et al. 2005, Zeng et al. 2008a). Accordingly, Jiang et al. showed recently (2010) that HO-1 overexpression increased the survival of transplanted MSCs in the infarcted swine hearts at week 1 post-MI. However, the MSCs were mostly engulfed by cardiac macrophages, and no MSCs were detected at 3 months post-MI (Jiang et al. 2010). HO-1-transfected MSCs decreased infarct size, improved left ventricular function, and increased microvascular density in postinfarction rat and swine hearts (Zeng et al. 2008a, Jiang et al. 2010, Tsubokawa et al. 2010). Although HO-1 increased survival of MSCs in the infarcted hearts to some extent, the beneficial effect of HO-1-modified MSCs was more likely mediated by paracrine factors secreted by these cells. Zeng et al. (2008b) showed that HO-1 increases production of growth factors and cytokines, including hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and VEGF, in MSCs *in vitro*, and injection of cell culture supernatants of HO-1 transfected MSCs in the infarcted rat hearts improved cardiac function, decreased infarct size and increased microvessel density. Furthermore, Lin et al. (2008) showed that cardiac HO-1 gene transfer promotes neovascularization in the ischemic mouse hearts by up-regulating the angiogenic factors VEGF and SDF-1 and recruitment of c-kit+ and CD34+ circulating stem/progenitor cells.

HO-1 gene therapy is a potential therapeutic strategy for protecting the heart against ischemic injury and promoting the healing of infarcted hearts. Pachori et al. (2004) showed that HO-1 gene delivery by a hypoxia-regulated viral vector 5 weeks prior to I/R injury improved cardiac function, reduced infarct size, and decreased expression of proinflammatory cytokines in the infarcted rat hearts. Likewise, HO-1 gene delivery by a hypoxia-regulated plasmid system improved the recovery of cardiac function and protected against ischemic injury in infarcted mouse hearts (Tang et al. 2005). It has been speculated that prolonged HO-1 overexpression may have adverse toxic effects. The hypoxia-regulated gene delivery system alleviates the possible toxic effects of high HO-1 levels, because it is active only in ischemic conditions. The pre-emptive HO-1 gene delivery also improved cardiac function, reduced myocardial injury, and prevented adverse left ventricular remodeling 12 days after repeated episodes of I/R or 1.5 and 3 months after a single episode of I/R in rat hearts (Liu et al. 2006, Pachori et al. 2006). Furthermore, the pre-emptive HO-1 gene delivery provided long-term protection by increasing survival of rats, improving cardiac function, and reducing post-MI left ventricular remodeling and heart failure 1 year after MI (Liu et al. 2007).

HO-1 also protects against pathologic left ventricular remodeling. Recently, Wang et al. (2010) demonstrated that cardiac-specific HO-1 overexpression in mice has several beneficial effects in failing hearts. HO-1 improved post-MI survival, ameliorated left ventricular dilatation and dysfunction, decreased apoptosis, hypertrophy, interstitial fibrosis, and oxidative stress, and increased neovascularization (Wang et al. 2010). They also showed that the beneficial effects were mediated at least partially by CO (Wang et al. 2010). Likewise, HO-1 gene therapy decreased apoptosis, interstitial fibrosis, and accumulation of myofibroblasts in the infarcted rat hearts (Liu et al. 2006, 2007, Pachori et al. 2006). In addition, HO-1 induction by hemin attenuated left ventricular hypertrophy and fibrosis in adult spontaneously hypertensive rats (Ndisang and Jadhav 2009), and HO-1 induction by CoPPIX inhibited angiotensin II-induced cardiac hypertrophy in rats (Hu et al. 2004). Furthermore, HO-1 contributed to improved ventricular function and decreased hypertrophy and interstitial fibrosis in mice lacking Bach1, the transcriptional repressor of HO-1 (Mito et al. 2008).

HO-1 is induced by numerous factors and mediates the beneficial effects of many of these factors. Resveratrol promotes neovascularization in infarcted rat hearts, in part via HO-1 (Kaga et al. 2005). Likewise, Samuel et al. showed recently (2010) that the proangiogenic effect of thioredoxin-1 is mediated by HO-1 in infarcted rat hearts. HO-1 also contributes to the

cardioprotective effects of inducible NO synthase (iNOS), erythropoietin, and Hif-1 (Ockaili et al. 2005, Burger et al. 2009, Li et al. 2009).

Studies investigating HO-1 in cardiac patients are relatively few. Corradi et al. (2008) showed increased expression of HO-1 in left atrial myocardium of patients with chronic atrial fibrillation. HO-1 expression was higher in the left atrial areas with greater structural remodeling (Corradi et al. 2008). Myocardial HO-1 expression appears to increase in various cardiac diseases in response to stress. Grabellus et al. (2002) showed increased expression of HO-1 in the myocardium of patients with end-stage heart failure, whereas left ventricular mechanical support by a left ventricular assist device significantly decreased myocardial HO-1 expression. Furthermore, HO-1 expression was markedly increased in the myocardial tissue of a patient with increased production of ROS due to mitochondrial cardiomyopathy (Ishikawa et al. 2005). HO-1 expression has also been studied in peripheral blood monocytes and lymphocytes of patients with coronary heart disease (Chen et al. 2005, 2009, Ishikawa et al. 2005). HO-1 levels were increased in patients with coronary heart disease, and the levels correlated with the severity of disease, with the highest levels in patients with acute MI (Chen et al. 2005, 2009).

The increase in HO-1 is likely a protective response in patients, since higher serum bilirubin levels associated with decreased risk for coronary artery disease (Hopkins et al. 1996). Serum total bilirubin levels are also inversely correlated with the prevalence of metabolic syndrome among children and adolescents (Lin et al. 2009). The protective effect of higher serum bilirubin levels against ischemic heart disease was also described in patients with Gilbert syndrome, a genetic disorder characterized by above-normal levels of circulating unconjugated bilirubin (Vitek et al. 2002). Conversely, low serum bilirubin concentrations were associated with coronary artery calcification (Tanaka et al. 2009).

In addition, *HO-1* polymorphisms in humans may modulate the susceptibility to coronary artery disease. It has been suggested that the short GT_n repeat length protects against coronary artery disease (Chen et al. 2002, Kaneda et al. 2002, Dick et al. 2005, Brydun et al. 2007, Chen et al. 2008). However, in some studies, especially in the large study of over 3000 patients by Lublinghoff et al. (2009), the association between *HO-1* polymorphisms and coronary artery disease was not found (Endler et al. 2004, Holweg et al. 2005, Lublinghoff et al. 2009).

2.4.2 HO-1 in atherosclerosis and vascular injury

The increased production of ROS and vascular inflammation lead to the development of atherosclerosis. Increasing evidence shows that HO-1 protects against atherosclerotic vascular disease. HO-1 is induced by several proatherogenic factors, including oxidized low-density lipoprotein (LDL), increased blood pressure, and smoking (Wang et al. 1998, Ndisang and Wang 2003, Fukano et al. 2006). In addition, HO-1 is found in atherosclerotic lesions in both humans and animals, and it is detected at high levels in macrophages/foam cells of the lesions throughout development of the lesions (Wang et al. 1998, Ishikawa et al. 2001b). In the early stage of lesion formation, HO-1 is also expressed in endothelial cells, whereas in advanced lesions HO-1 is found in SMCs (Wang et al. 1998, Ishikawa et al. 2001b). HO-1 induction by hemin reduces lesion size in LDL-receptor knockout mice, whereas inhibition of HO-1 by SnPPIX increases atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits (Ishikawa et al. 2001a, 2001b). Furthermore, Yet et al. (2003) showed that mice deficient in both HO-1 and apolipoprotein E (ApoE) develop larger and more advanced lesions than mice deficient in ApoE only. HO-1 also reverses atherosclerotic plaque progression from vulnerable plaque to a more stable phenotype by reducing the size of the necrotic core and intraplaque lipid accumulation and increasing the thickness of the fibrous cap and accumulation of VSMCs in the intima in apoE-null mice (Cheng et al. 2009).

HO-1 modulates the development of atherosclerosis by multiple mechanisms. HO-1 induction by hemin reduces monocyte chemotaxis in response to oxidized LDL (Ishikawa et al. 1997). In addition, Orozco et al. (2007) showed that HO-1 expression in macrophages is important for the antiatherogenic effect of HO-1. They showed that both reduced or absent HO-1 expression in macrophages increased generation of ROS, production of inflammatory cytokines, and formation of foam cells when treated with oxidized LDL *in vitro* (Orozco et al. 2007). The absence of HO-1 also resulted in greater macrophage content in atherosclerotic lesions *in vivo* (Orozco et al. 2007). Increased iron deposition is present in atherosclerotic lesions and is associated with the progression of atherosclerosis in ApoE-deficient mice (Lee et al. 1999). HO-1 may protect against excess vascular iron deposition, since HO-1-deficient mice have increased tissue iron accumulation (Poss and Tonegawa 1997b). Conversely, HO-1 prevents cell death by enhancing cellular iron efflux in mouse fibroblasts (Ferris et al. 1999). In addition, Juan et al. (2001) showed that HO-1 gene transfer indeed decreases iron deposition in the atherosclerotic lesions of ApoE-deficient mice.

The protective role of bilirubin against atherosclerosis and coronary artery disease was observed in 1994. Wu et al. (1994) showed that bilirubin efficiently prevents oxidation of LDL, and Schwertner et al. (1994) reported that low serum bilirubin concentration is associated with increased risk of coronary artery disease. The inverse correlation between serum bilirubin levels and the risk for coronary artery disease was confirmed later in several studies (Hopkins et al. 1996, Vitek et al. 2002). Bilirubin and biliverdin are efficient scavengers of oxidants, and they may protect the vasculature by their antioxidant action (Stocker and Peterhans 1989). Bilirubin protects VSMCs against oxidative stress and inhibits lipid peroxidation in LDL-receptor knockout mice, as demonstrated by decreased plasma lipid hydroperoxide (LPO) levels (Clark et al. 2000a, Ishikawa et al. 2001b). Kawamura et al. (2005) showed that HO-1-derived bilirubin suppresses production of proinflammatory mediators and attenuates endothelial dysfunction. In addition, bilirubin decreases monocyte transmigration in response to oxidized LDL (Ishikawa et al. 1997). Bilirubin and biliverdin also reduce proliferation of SMCs *in vitro* and decrease balloon injury-induced neointima formation in hyperbilirubinemic Gunn rats (Ollinger et al. 2005).

CO potentially contributes to the antiatherogenic effect of HO-1 by several mechanisms, of which the anti-inflammatory and antiproliferative effects are likely the most important. Otterbein et al. (2003b) showed that CO suppresses arteriosclerotic lesions associated with chronic graft rejection by inhibiting leukocyte infiltration, SMC proliferation, and expression of proinflammatory cytokines in the transplanted aortic segments. They also showed that CO reduced neointimal hyperplasia and completely prevented vascular stenosis after balloon angioplasty (Otterbein et al. 2003b). Recently, Hyvelin et al. (2010) showed that HO-1 induction by hemin reduced in-stent stenosis in rats and rabbits and a similar effect was observed in rats after treatment with CO donor CORM-2. The anticoagulative, antiapoptotic, and vasodilatory effects of CO may also contribute to the protection against atherosclerosis and vascular injury. CO inhibits human platelet aggregation *in vitro* and platelet-dependent thrombosis in mice (Brune and Ullrich 1987, Peng et al. 2004). In addition, CO suppresses rejection of mouse-to-rat cardiac transplants by inhibiting platelet aggregation, thrombosis, and endothelial cell apoptosis (Sato et al. 2001).

2.4.3 HO-1 in hypertension

Numerous studies have shown that HO-1 has antihypertensive effects via its reaction product CO. HO-1 is up-regulated in aortic tissue of spontaneously hypertensive rats, and in response to angiotensin II in many cells and organs, including VSMCs, endothelial cells, heart and kidneys

(Ishizaka and Griendling 1997, Aizawa et al. 2000, Ishizaka et al. 2000, Mazza et al. 2003, Cheng et al. 2004). Administration of heme, heme derivatives, or CO reduces blood pressure of hypertensive rats (Levere et al. 1990, Johnson et al. 1996, Ndisang et al. 2002). Accordingly, HO-1 gene transfer reduces blood pressure in spontaneously hypertensive rats and prevents angiotensin II-induced increase in blood pressure (Sabaawy et al. 2001, Yang et al. 2004). Botros et al. (2005) showed that HO-1 induction by CoPPIX decreases blood pressure in a renovascular hypertension model, whereas HO-1 inhibition by tin mesoporphyrin (SnMP) elevates blood pressure. Furthermore, Wiesel et al. (2001) showed in the same renovascular hypertension model that absence of HO-1 leads to more severe renovascular hypertension and cardiac hypertrophy. Interestingly, Wang et al. (2006) showed in spontaneously hypertensive rats that administration of hemin for 3 weeks by osmotic minipumps caused increased HO-1 expression and activity and normalized systolic blood pressure, and these changes were maintained for 9 months after hemin treatment. In contrast to these antihypertensive effects, HO-1 and CO promote endothelial dysfunction and contribute to salt-induced hypertension in Dahl salt-sensitive rats and deoxycorticosterone acetate (DOCA) salt-sensitive rats (Johnson et al. 2003, 2004, Teran et al. 2005). However, despite the skeletal muscle endothelial dysfunction in Dahl rats, HO-1 and CO protected the heart by promoting coronary vasodilatation in the same animals (Johnson et al. 2004).

3 AIMS OF THE STUDY

The aim of the present study was to investigate the role of HO-1 in protection and repair of the heart, examine the potential mechanisms mediating the cardioprotective and prohealing effects of HO-1 in the experimental rat models, and to evaluate the expression and significance of HO-1 in critically ill patients.

The specific aims were:

1. To investigate the expression and function of HO-1 in postinfarction rat hearts and determine which cells express HO-1 in the heart. (I–III)

2. To determine the long-term and short-term cardioprotective effects of HO-1 induction in infarcted rat hearts and in isolated I/R rat hearts. (Studies II-IV)

3. To determine the roles of HO-1 induction and CO donor pretreatment in postinfarction cardiac regeneration. (II)

4. To determine the roles of HO-1 induction and CO donor pretreatment in cellular and extracellular remodeling in the infarcted rat hearts. (III)

5. To determine the association of *HO-1* polymorphisms and HO-1 plasma concentrations with disease severity (clinical scores), degree of organ dysfunction and mortality in critically ill patients, and evaluate the role of HO-1 in the subgroup of cardiac patients. (V)

4 MATERIALS AND METHODS

4.1 Animals

A total of 334 adult male Wistar rats were used in the experimental studies (I–IV). Table 4 shows the summary of rat numbers and groups (I–IV).

4.2 Experimental myocardial infarction

4.2.1 Experimental protocol 1: HO-1 expression in postinfarction hearts (I)

MI was produced by permanent occlusion of the left anterior descending (LAD) coronary artery. A total of 98 adult male Wistar rats (weight 350–500 g) were used in the experiment. The rats were anesthetized, using medetomidine (0.5 mg/kg subcutaneously (s.c.), Domitor®; Orion, Turku, Finland) and ketamine (70–80 mg/kg intraperitoneally (i.p.), Ketalar®; Parke Davis, Barcelona, Spain), connected to a rodent respirator through a tracheotomy, and ventilated with oxygen-supplemented air. A thermal plate was used to maintain stable body temperature during the operation. The heart was exteriorized through a left thoracotomy, the pericardium was opened, and the proximal LAD was ligated. After the coronary ligation, the heart was returned to its normal position, the thorax was closed, and the rats were given atipamezole hydrochloride (0.75 mg/kg s.c., Antisedan®; Orion) to partially antagonize the anesthesia, physiological saline (10 ml s.c.) for hydration, and buprenorphin (0.02 mg/kg s.c., Temgesic®, Reckitt & Colman, Hull, UK) twice for postoperative analgesia. The number of MI rats was 58. The sham operated control rats underwent the same procedure, except for the ligation of the coronary artery (n = 40). The rats were killed with CO_2 1 day, 1 week, and 4 weeks after LAD ligation. The number of rats in each group is shown in Table 4. The heart was excised and cut into 2-mm transverse slices that were immediately frozen in liquid nitrogen and stored at –70 °C or fixed in 4% neutral buffered formalin for 24 h and embedded in paraffin.

4.2.2 Experimental protocol 2: Effects of pre-emptive HO-1 induction and CO donor pretreatment on recovery of myocardial infarction (II, III)

LAD ligation or the sham operation was performed in a total of 180 adult male Wistar rats (weight 280–380 g). To achieve maximal HO-1 expression and CO levels at the time of LAD ligation, the rats were pretreated with a single dose of the HO-1 inducer CoPPIX (5 mg/kg i.p.; Frontier Scientific, Logan, UT, USA) 24 h prior to LAD ligation (n = 60) or a single dose of CO donor methylene chloride (MC, 500 mg/kg perorally (p.o.); Sigma, St. Louis, MO, USA) 3 h prior to LAD ligation (n = 60), whereas control rats (n = 60) received no pretreatment. The rats were anesthetized, using medetomidine (0.5 mg/kg s.c.) and ketamine (60 mg/kg s.c.), and mechanically ventilated as described above. The heart was exposed through a lateral thoracotomy and the coronary artery was ligated about 3 mm from its origin. The successful ligation was confirmed by visual inspection of the pale color in the occluded distal myocardium. Postoperatively, the rats were hydrated as described above and given buprenorphin (0.05 mg/kg s.c.) twice daily for 3 days for analgesia. In all, 16 rats died in the course of the experiment (mortality rate 9%). The remaining rats were anesthetized with sodium pentobarbital (60 mg/kg i.p., Mebunat Vet®; Orion) 1 day, 3 days, 1 week, or 4 weeks after coronary ligation. Blood samples were drawn from the jugular vein and the blood carboxyhemoglobin (COHb) was measured by CO oximetry, using the ABL800 FLEX Analyzer (Radiometer, Copenhagen, Denmark). The heart was excised and weighed, and the myocardial samples were processed as

described above. The number of rats in each group is shown in Table 4. The induction of HO-1 was confirmed by increased HO-1 mRNA and protein levels (study II, Fig. 1 and study III, Table 1). The success of CO donor pretreatment was confirmed by the higher COHb levels at day 1 (study II, Fig. 1).

Table 4. Summary of the rats (total n = 334) used in studies I-IV.

Study	Model	Total n of rats used in the experiment	Groups	Final n per group
I	Experimental MI	98	1 day MI	16
			1 day sham	13
			1 week MI	24
			1 week sham	15
			4 weeks MI	18
			4 weeks sham	12
II and III	Experimental MI	180	1 day HO-1-induction MI	7
			1 day CO MI	8
			1 day control MI	8
			1 day HO-1 induction sham	5
			1 day CO sham	5
			1 day control sham	5
			3 days HO-1-induction MI	8
			3 days CO MI	7
			3 days control MI	8
			3 days HO-1 induction sham	5
			3 days CO sham	5
			3 days control sham	5
			1 week HO-1-induction MI	7
			1 week CO MI	7
			1 week control MI	7
			1 week HO-1 induction sham	5
			1 week CO sham	5
			1 week control sham	5
			4 weeks HO-1-induction MI	9
			4 weeks CO MI	10
			4 weeks control MI	8
			4 weeks HO-1 induction sham	5
			4 weeks CO sham	5
			4 weeks control sham	5
IV	Isolated rat hearts (I/R-model)	56	HO-1 induced I/R (Group 1a)	8
			HO-1 induced control (Group 1b)	8
			ZnPPIX I/R (Group 2a)	8
			ZnPPIX control (Group 2b)	8
			Non-pretreated I/R (Group 3a)	8
			Non-pretreated control (Group 3b)	8
			Baseline control	8

Abbreviations: CO = carbon monoxide, HO-1 = heme oxygenase-1, I/R = ischemia/reperfusion, MI = myocardial infarction, ZnPPIX = zinc protoporphyrin IX. Studies II and III: the rats that died during the experiment (n = 16) and the hearts with no histological signs of MI (n = 10) are not included in the final number of rats/hearts per group.

4.3 Isolated rat heart preparation (IV)

A total of 56 adult male Wistar rats weighing 250–300 g were divided into six experimental groups and one baseline control group (n = 8 in each group). Groups 1a and 1b received no pretreatment. Groups 2a and 2b were given the HO-1 inducer hemin (50 μmol/kg i.p.; Sigma) 24 h before excision of the hearts and groups 3a and 3b were given the HO enzyme inhibitor ZnPPIX (50 μmol/kg i.p.; Aldrich Chemical Co., Milwaukee, WI, USA) 1 h before excision of the hearts. The rats were anesthetized with diethyl ether and given heparin (500 U/kg) intravenously (i.v.) as an anticoagulant. The hearts were rapidly excised, mounted in a perfusion system, (Fig. 5B) and perfused in the Langendorff or working mode (Fig. 5A) with modified normothermic Krebs-Henseleit buffer (118.4 mM NaCl, 4.1 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.17 mM KH_2PO_4, 1.46 mM $MgCl_2$ and 11.1 mM glucose, pH 7.4 at 37 ºC)(Ferdinandy et al. 1993, Csonka et al. 1999). The perfusion buffer was saturated with 95% O_2 and 5% CO_2. The hearts were equilibrated in the working mode for 15 min, followed by 30 min of global no-flow ischemia and 120 min of reperfusion (groups 1a, 2a, and 3a). Time-matched nonischemic control hearts were aerobically perfused for a total of 165 min (groups 1b, 2b, and 3b). A ventricular pressure curve was obtained, using a small polyethylene catheter that was placed in the left ventricle through the left atrial cannula and connected to a pressure transducer (Ferdinandy et al. 1993, Csonka et al. 1999). Five min before ischemia and at the 15th min of reperfusion, the heart rate (HR), aortic flow (AF), coronary flow (CF), left ventricular developed pressure (LVDP), positive and negative first derivetives of left ventricular pressure (+dp/dt and -dp/dt) and left ventricular end-diastolic pressure (LVEDP) were monitored (Ferdinandy et al. 1993, Csonka et al. 1999). After recording the postischemic hemodynamic parameters, the perfusion was switched to the Langendorff mode. An epicardial electrocardiogram (ECG) was monitored throughout the experiments by two silver electrodes attached directly to the heart to determine the incidence of arrhythmias. Normal sinus rhythm was characterized by normal sinus complexes occurring in a regular rhythm on the ECG, whereas an irregular undulating baseline on the ECG was considered to represent VF (Fig. 5C). The cardiac functional parameters and ECG were also recorded from time-matched nonischemic control hearts. At the end of each experiment, the ventricles were cut into 2-3-mm slices and frozen in liquid nitrogen for subsequent analyses. In addition, one group of animals was killed at baseline (0-min time point) and myocardial samples collected to serve as nonperfused baseline controls for mRNA, protein, and cGMP measurements. The experimental design is shown in study IV, Fig. 1. The effects of hemin and ZnPPIX pretreatments on HO-1 mRNA and protein expression are shown in study IV, Figs. 2 and 3.

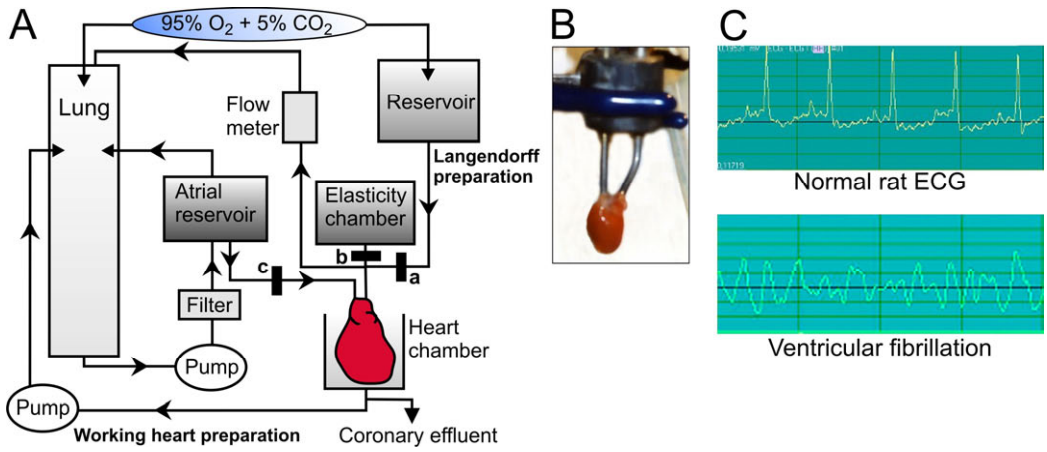

Figure 5. Isolated rat heart perfusion system. A) Simplified diagram of the perfusion system. In the Langendorff mode tap a is open (taps b and c are closed). In the working heart mode tap a is closed and taps b and c are open. B) The heart cannulated via the aorta and the left atrium. C) Representative rat ECGs of normal sinus rhythm and ventricular fibrillation. Abbreviations: ECG = electrocardiogram.

4.4 Real-time quantitative RT-PCR (I–IV)

Total RNA was isolated from rat heart tissues, using Trizol reagent (Invitrogen/Gibco BRL, Grand Island, NY, USA) and reverse-transcribed to cDNA, using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen/Gibco BRL). The cardiac gene expression was studied by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), using either the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) or the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The PCR reactions were performed in duplicate or triplicate in a 25-μl final volume using either SYBR Green or TaqMan chemistry. The primer and probe sequences are shown in Table 5. The TaqMan Gene Expression Assay primer and probe mix was used for controlling gene 18S (Applied Biosystems, part # Hs999999_s1). A dilution series of purified PCR products was used to generate standard curves and the data were analyzed using the absolute standard curve method (described in the Applied Biosystems User Bulletin #2, 1997; www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). Either 18S or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control gene for normalizing the mRNA copy numbers. The control gene was selected for each study so that the expression of the control gene did not differ significantly among the experimental groups.

Table 5. Primers and probes used in gene expression analyses.

mRNA	Sequence	Used in
ANP		
Forward primer	5'-AGCGGACTAGGCTGCAACAG-3'	III
Reverse primer	5'-TGGCAATGCGACCAAGCT-3'	
BNP		
Forward primer	5'- TCGAAATTCCAAGATGGCACAT-3'	-
Reverse primer	5'- GTCACAGCCCAAGCGACTGA-3'	
Coll1a1		
Forward primer	5'-AGCGTGCTGTAGGTGAATCGA-3'	III
Reverse primer	5'-TGCAAGAACAGCGTAGCCTACAT-3'	
Coll3a1		
Forward primer	5'-TTGGGATGCAACTACCTTGGT-3'	III
Reverse primer	5'-CCCGAGTCGCAGACACATATT-3'	
CTGF		
Forward primer	5'-GTGTGTGATGAGCCCAAGGA-3'	III
Reverse primer	5'-GCAGTTGGCTCGCATCATAG-3'	
Probe	6-FAM-CCAAATGTGTCTTCCAG-MGB	
Fibronectin		
Forward primer	5'-GGCAACAAATGATCTTTGAGGAA-3'	III
Reverse primer	5'-TCTACATTCGGCAGGTATGGTCTT-3'	
HO-1		
Forward primer	5'-CACAAAGACCAGAGTCCCTCACAG-3'	I, II, III, IV
Reverse primer	5'-AAATTCCCACTGCCACGGT-3'	
HO-2		
Forward primer	5'-CCATGGCTGTGCTGAGGAA-3'	I, IV
Reverse primer	5'-GGTCACTTGGGATAGGATGCA-3'	
TGF-β1		
Forward primer	5'-GAAGTCACCCGCGTGCTAAT-3'	III
Reverse primer	5'-GCTTCCCGAATGTCTGACGTA-3'	

4.5 Histology and infarct size (II, III)

Several 5-μm transverse paraffin sections from the midventricular level of rat hearts were used for all histological analyses. The presence of MI was determined from tissue sections stained with Weigert van Gieson. Hearts with no histological signs of infarction (eosinophilia, karyolysis, and leukocyte infiltration indicative of acute infarction or collagen scars compatible with old infarction) were not included in the study (n = 10). Infarct size was determined planimetrically as the ratio of infarcted tissue or scar to the length of the entire endocardial circumference of the left ventricle (Pfeffer et al. 1979). Toluidine blue staining was used to identify mast cells. Interstitial and perivascular fibrosis were analyzed from tissue sections stained with collagen-specific picrosirius red. Interstitial fibrosis was measured, using ImageJ software. In all, 6–10 randomly selected microscopic fields (100x or 200x magnification) photographed from the noninfarcted remote myocardium were analyzed. The extent of interstitial fibrosis was expressed as the percentage of fibrosis of the total left ventricular area. Perivascular fibrosis was also measured by using ImageJ software. A total of 6–15 vessels per heart were measured and the extent of perivascular fibrosis was expressed as the ratio of fibrotic area surrounding the vessel to the total vessel wall area. Apoptosis of the cardiomyocytes was determined, using terminal transferase-mediated DNA nick-end labeling (TUNEL) (Saraste et al. 1997). To quantitatively assess the number of TUNEL+ cardiomyocytes, the proportions of the infarct area, border area, and remote myocardium of the total area of the ventricular sections were determined and the number of cardiomyocytes per mm^2 of border area and remote myocardium was counted. Viable myocardium extending 0.5–1 mm from the infarct area was considered to represent the infarct border area. The number of apoptotic cardiomyocytes was expressed as the percentage of TUNEL+ cardiomyocytes in the total number of cardiomyocytes.

4.6 Immunohistochemical analyses (I–IV)

Series of 5-μm transverse paraffin sections or 7-μm transverse cryosections from the midventricular level of rat hearts were used for imunohistochemical analyses. Immunofluorescent staining was performed to examine HO-1 expression and localization in the infarcted rat hearts, the effects of HO-1 and CO on cardiac regeneration and ventricular remodeling in infarcted rat hearts, and the effect of HO-1 induction or inhibition on connexin 43 (Cx43) expression and localization in I/R rat hearts. The primary antibodies are listed in Table 6. The secondary antibodies were: Alexa Fluor 488-conjugated goat antimouse or donkey antigoat IgG (Molecular Probes, Eugene, OR, USA), Alexa Fluor 546-conjugated goat antirabbit IgG (Molecular Probes), and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated swine antirabbit IgG (Dako Cytomation, Glostrup, Denmark). We used double stranded DNA-binding 4´6-diamino-2-phenylindole (DAPI, Molecular Probes) as a nuclear counterstain. The immunolabeled sections were examined, using Leica TCS 4D confocal system connected to a Leica RXA microscope (Leica Lasertechnik, Heidelberg, Germany), Leica DM 4500B fluorescent microscope (Leica Microsystems, Wetzlar GmbH, Wetzlar, Germany), or a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Cardiac regeneration was assessed by counting the number of c-kit+ stem/progenitor cells, vWF+ capillaries and α-smooth muscle actin (α-SMA+) vessels in the infarcted hearts. The following markers were used to evaluate proliferation and differentiation of c-kit+ cells: Ki67 (proliferation marker), Nkx2.5 (early cardiomyocyte transcription factor), GATA6 (SMC transcription factor), Ets-1 (endothelial cell transcription factor), and CD34 (hematopoietic stem cell marker). The vascular and capillary densities were analyzed from 6 to 15 randomly selected microscopic fields (200x or 400x magnification) photographed from each of the infarct, border, and remote areas.

Table 6. Primary antibodies used in immunohistochemistry and western blotting.

Protein	Antibody	Manufacturer	Application	Used in
Actin	rabbit polyclonal	Sigma	W	II, III, IV
α-SMA	mouse monoclonal	Dako Cytomation	IHC	II, III
CD34	goat polyclonal	R&D Systems	IHC	II
c-kit	rabbit polyclonal	Dako Cytomation	IHC	II
Cx43	rabbit polyclonal	Zymed	IHC, W	IV
Cx43	mouse monoclonal	Zymed	IHC, W	IV
Ets-1	mouse monoclonal	NeoMarkers	IHC	II
GATA6	mouse monoclonal	R&D Systems	IHC	II
HIF-1α	mouse monoclonal	Sigma	W	II
HO-1	rabbit polyclonal	Stressgen	IHC, W	I, IV
H3P	rabbit polyclonal	Cell Signaling Technology	IHC	III
Ki67	rabbit monoclonal	NeoMarkers	IHC	II, III
Ki67	mouse monoclonal	Dako Cytomation	IHC	II
MHC	mouse monoclonal	Upstate	IHC	II, III
Nkx2.5	mouse monoclonal	R&D Systems	IHC	II
PCNA	mouse monoclonal	Cell Signaling Technology	IHC	III
Procol I	goat polyclonal	Santa Cruz	W	III
Spectrin	mouse monoclonal	Chemicon	W	II
cTnI	rabbit polyclonal	Santa Cruz	IHC	III
VEGF-A	rabbit polyclonal	Millipore	W	II
VEGF-B	mouse monoclonal	R&D Systems	IHC, W	II
Vimentin	mouse monoclonal	NeoMarkers	IHC, W	IV
vWF	rabbit polyclonal	Chemicon	IHC	II

Abbreviations: α-SMA = α-smooth muscle actin, Cx43 = connexin 43, HIF-1α = hypoxia inducible factor-1α, HO-1 = heme oxygenase-1, H3P = phosphorylated histone H3, IHC = Immunohistochemistry, MHC = cardiac myosin heavy chain, PCNA = proliferating cell nuclear antigen, Procol I = procollagen type I, cTnI = cardiac troponin I, VEGF = vascular endothelial growth factor, vWF = von Willebrand factor, W = Western blotting.

Proliferation of cells in the infarct area, border area, and remote myocardium was assessed by counting the proportion of Ki67+ nuclei in comparison to the total count of nuclei per mm^2. Proliferation of cardiomyocytes was determined by counting the number of Ki67 and cardiac myosin double-positive cells throughout the infarct border area and remote myocardium, and expressed as the percentage of Ki67+ cardiomyocytes out of the total number of cardiomyocytes. The total number of cardiomyocytes in the infarct border area and remote myocardium were determined, as described above for cardiomyocyte apoptosis. Proliferation of fibroblasts was assessed by counting the number of Ki67 and vimentin double-positive fibroblastlike cells per mm^2. The total number of vimentin+ cells per mm^2 having the characteristic morphology of fibroblasts was counted from six randomly selected microscopic fields (200x or 400x magnification) photographed from each of the infarct, border, and remote areas. Proliferation of fibroblasts was expressed as the percentage of Ki67+ fibroblasts out of the total number of fibroblasts.

Repair of cardiomyocyte DNA was analyzed, using a marker for DNA synthesis and repair: proliferating cell nuclear antigen (PCNA) (Savio et al. 1998). Repair of cardiomyocytes was assessed by counting the number of PCNA and cardiac troponin I double-positive cells throughout the infarct border area and remote myocardium, and expressed as the percentage of PCNA+ cardiomyocytes out of the total number of cardiomyocytes. The total number of

cardiomyocytes in the infarct border area and remote myocardium were determined, as described above for cardiomyocyte apoptosis.

Cardiomyocyte size was analyzed by measuring the cross-sectional area of 100 cardiomyocytes per heart, using ImageJ software. The cardiomyocytes cut in the short axis with a visible nucleus were measured from tissue sections stained with anticardiac myosin and DAPI.

4.7 Western blotting and ELISA (I–IV)

Protein levels in the rat heart tissue were studied, using Western blotting and enzyme-linked immunosorbent assay (ELISA). Homogenization of heart tissue and Western blotting are described in detail in the original publications (I–IV). The primary antibodies used in the Western blotting are listed in Table 6. Detection was performed, using the SuperSignal West Pico Chemiluminescent System (Pierce, Rockford, IL, USA) or Opti-4CN Detection kit (BioRad Laboratories, Hercules, CA, USA). Signal intensities were quantified, using Gel Doc Image Analyzer (BioRad).

Cardiac HO-1 protein was measured from homogenized heart tissues, using a StressXpress Rat HO-1 ELISA kit (Assay Designs/Stressgen (now Enzo Life Sciences), Victoria, BC, Canada) and a Multiskan EX Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). HO-1 protein levels were measured from sham-operated hearts and from the infarct, infarct border, and remote areas of infarcted hearts, and reported as ng of HO-1 protein per mg of total protein. Total protein in the heart tissue homogenates was measured, using the BCA protein assay kit (Pierce). A Quantikine mouse SDF-1α Elisa kit (R&D Systems, Minneapolis, MN, USA) was used to measure SDF-1α protein levels in the heart tissue homogenates. The SDF-1α protein levels were measured from the infarct areas of infarcted hearts and sham-operated hearts. Absorbances were measured at 450 nm, using Multiskan EX Microplate Photometer, and the results were expressed as pg of SDF-1α protein per mg of total protein.

4.8 Measurement of cyclic GMP (IV)

The cGMP was extracted from frozen heart tissue, using the trichloroacetic acid (TCA) method (IV) and measured, using a competitive immunoassay (cGMP enzyme immunoassay kit, Sigma).

4.9 Statistical analyses: experimental studies (I–IV)

The results of continuous variables are shown as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using SPSS software (versions 9.0, 13.0, or 16.0; SPSS Inc., Chicago, IL, USA). Differences between the two groups were analyzed by Student's t-test. Multiple groups were compared, using either parametric one-way analysis of variance (ANOVA) followed by a Bonferroni or Tukey test, or nonparametric Kruskall-Wallis test followed by the Mann-Whitney *U*-test. Differences between categorical variables (the incidence of VF) were compared, using the chi-square test. The differences were considered significant at a P value of < 0.05.

4.10 Clinical study (V)

4.10.1 Patients and study design

This is a prospective cohort study of unselected critically ill patients admitted to one medical and two medical-surgical intensive care units (ICUs) in Helsinki University Central Hospital between January 2004 and July 2005. A total of 244 patients were enrolled after obtaining written informed consent from patients or their authorized representatives; 13 patients were excluded due to inadequate blood samples. The final study population was 231 patients. The baseline characteristics of the final study population and grouping of the study population according to the International Classification of Diseases 10th edition (ICD-10) are shown in Tables 7 and 8. In addition, plasma samples of 58 healthy control subjects (28 women and 30 men), a subpopulation of the Nordic reference interval project, were used to determine the reference range for plasma HO-1 (Rustad et al. 2004).

The HO-1 concentrations were measured in serial plasma samples and *HO-1* polymorphisms determined. The association of HO-1 plasma concentrations and *HO-1* polymorphisms with ICU and hospital mortality, degree of organ dysfunction, illness severity, bilirubin, and C-reactive protein (CRP) was investigated.

Table 7. Baseline characteristics of the study patients (n = 231).

	Survivors (n= 186)	Nonsurvivors (n=45)	p value
Age	57 (47-66)	64 (52-73)	0.009
Gender (male)	115 (62%)	38 (84%)	0.005
Length of ICU stay (days)	3.7 (1.9-7.6)	3.8 (2.2-8.8)	0.43
Chronic disease	28 (15%)	13 (29%)	0.04
First day SOFA score	7.5 (5-10)	11 (7-13)	<0.001
Maximum SOFA score	8 (6-10)	11 (9-13)	<0.001
Multiple organ dysfunction	116 (62%)	38 (84%)	0.006
APACHE II score	22 (16-28)	28 (23-34.5)	<0.001
SAPS II score	42 (29-52)	57 (46-69)	<0.001

The data are given in median (interquartile range) in continuous variables and in count (percents) in categorical variables. Abbreviations: APACHE II = acute physiology and chronic health evaluation II score, ICU = intensive care unit, SAPS II = simplified acute physiology score II, SOFA = sequential organ failure assessment score.

4.10.2 Blood samples and data collection

Three blood samples were collected in 10-ml ethylenediaminetetraacetic acid (EDTA) tubes. The first blood sample was drawn as soon as possible after ICU admission (day 1 sample, n = 160), the second on the following morning (day 2 sample, n = 162), and the third 48 h after the second sample (day 3–4 sample, n = 187). Plasma was separated as soon as possible by centrifugation at 1600g (+4 ºC) for 10 min and stored at -80 ºC. The bilirubin and CRP values were obtained from the laboratory database, since they were measured daily as a routine follow-up of the patients. The patient data were collected and stored in the Finnish Intensive Care Quality Consortium's database (Intensium, Kuopio, Finland). The data included demographic factors, diagnosis by ICD-10, ICU and hospital mortality, and illness severity scores calculated within the first 24 h in the ICU: Simplified Acute Physiology Score (SAPS) II (Le Gall et al. 1993) and

Acute Physiology and Chronic Health Evaluation (APACHE) II score (Knaus et al. 1985), the degree of organ dysfunction calculated daily in the ICU: Sequential Organ Failure Assessment (SOFA) score (includes separately the status of cardiovascular, respiratory, hepatic, and renal systems, central nervous system, and coagulation as shown in Table 9)(Vincent et al. 1998).

Table 8. Grouping of the study population (n=231) according to ICD-10 diagnosis.

Diagnosis	n
Heart arrest, ventricular fibrillation	37
Respiratory tract infection, pneumonia	30
Sepsis	25
Heart infarct	22
Pancreatitis	16
Gastrointestinal bleeding	10
Heart failure	12
Intoxication	9
Meningitis, other bacterial infection	8
Cerebrovascular disorders	8
Convulsions	6
Acute renal failure	4
Liver failure	4
Diabetes, ketoacidosis	4
Other bacterial infection	4
Pulmonary embolism	3
Other	12
Operative	16

Abbreviations: ICD-10 = International Classification of Diseases 10th edition.

The variables included in the SAPS II score are: age (years), HR per min, systolic arterial pressure (mmHg), temperature (ºC), partial pressure of oxygen in arterial blood (PaO_2; mmHg or kPa)/fraction of inspired oxygen (FiO_2) if mechanically ventilated or with continuous positive airway pressure, serum HCO_3, white blood cell count (E9/l), urine output (l/24 h), serum urea (mmol/l) or serum urea nitrogen, serum bilirubin (μmol/l), serum sodium (mmol/l), serum potassium (mmol/l), Glasgow Coma Scale, type of admission (medical, scheduled, or unscheduled surgery), and underlying chronic disease (metastatic cancer, hematologic malignancy, or acquired immune deficiency syndrome) (Le Gall et al. 1993). The APACHE II score includes 12 physiological variables, age, and underlying severe chronic disease (Knaus et al. 1985). The physiological variables include HR per min, mean arterial pressure (mmHg), temperature (ºC), respiratory rate per min, oxygenation (FiO_2, mmHg), arterial pH (or serum HCO_3), hematocrit (%), white blood cell count (E9/l), creatinine (mg/100 ml), serum sodium (mmol/l), serum potassium (mmol/l), and Glasgow Coma Scale (Knaus et al. 1985).

Table 9. SOFA score.

	SOFA score				
Organ system	**0**	**1**	**2**	**3**	**4**
Cardiovascular *	MAP > 70 mmHg	MAP < 70 mmHg	DA ≤ 5 or dobutamine	DA > 5 or Epi ≤ 0.1 or NA ≤ 0.1	DA > 15 or Epi > 0.1 or NA > 0.1
Respiratory PaO2/FiO2 (mmHg)	≥ 400	< 400	< 300	< 200 (with mechanical ventilation)	< 100 (with mechanical ventilation)
Hepatic Bilirubin (μmol/l)	< 20	20-32	33-101	102-204	> 204
Renal Creatinine (μmol/l)	<110	110-170	171-299	300-440 or urine output < 500 ml/day	>440 or urine output < 200 ml/day
Central nervous system Glasgow Coma Scale	15	13-14	10-12	6-9	<6
Coagulation Platelet count (E9/l)	≥ 150	< 150	< 100	< 50	< 20

* Doses of adrenergic agents are given in μg/kg/min. Abbreviations: DA = dopamine, Epi = epinephrine, MAP = mean arterial pressure, NA = norepinephrine, SOFA = sequential organ failure assessment score.

4.10.3 *HO-1* genotyping

Genomic DNA was extracted from whole blood, using Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) and stored at -20 ºC. Genotyping of the GT_n repeat length polymorphism in the *HO-1* promoter was performed by DNA fragment analysis. The 5'-flanking region of the *HO-1* gene containing the GT_n repeats was amplified by PCR, using a fluorescently labeled forward primer and unlabeled reverse primer (primer sequences are shown in Table 10). The PCR reactions were performed in a 25-μl final volume containing 25 ng of genomic DNA, 300 nM of primers, 1 x AmpliTaq Gold PCR buffer (Applied Biosystems), 3 mM $MgCl_2$, 0.2 mM of each nucleoside triphosphate (NTP) (Applied Biosystems), and 0.625 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR cycling conditions were 10 min at +95 ºC and 40 cycles of 30 s at +95 ºC and 1 min 15 s at +54 ºC. The sizes of the labeled PCR products were determined, using an automated capillary sequencer (ABI3730xl DNA Analyzer, Applied Biosystems). A GeneScan-500 LIZ (Applied Biosystems) was used as a size marker. The number of GT_n repeats was calculated, using GeneMaker 1.4 software (SoftGenetics, State College, PA, USA). The GT_n repeat length genotype was obtained from 230 patients, because the DNA fragment analysis failed in one patient.

Genotyping of two SNPs, -413A/T (rs2071746) and +99G/C (rs2071747), was performed by allelic discrimination, using the 5' nuclease TaqMan assay. The primers and probes for -413A/T are shown in Table 10 (Ono et al. 2004). The TaqMan SNP Genotyping assay was used for +99G/C (Applied Biosystems). The PCR reactions for the +99G/C SNP were performed in a 10-μl final volume containing 10 ng of genomic DNA, 1 x TaqMan master mix (Applied Biosystems), and 1 x TaqMan SNP genotyping assay mix. The PCR reactions for -413A/T were performed in a 25-μl final volume containing 25 ng of genomic DNA, 1 x TaqMan master mix, 300 nM of forward and reverse primers, and 200 nM of both probes. The PCR reactions were performed,

using ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the Allelic Discrimination protocol. The PCR cycling conditions were 10 min at +95 ºC and 40 cycles of 20 s at +95 ºC and 1 min at +60 ºC. Negative controls with no template DNA were included in each PCR run. The genotypes were determined, using SDS 1.2 software (Applied Biosystems). Genotype assignment was performed manually and included examination of the amplification plot and raw fluorescence data. When necessary, samples were retested to confirm the genotype. The SNP genotypes were obtained from all 231 study patients.

Table 10. Primers and probes used in *HO-1* genotyping.

	Sequence
GT_n repeat length polymorphism	
GTn forward primer	5'-FAM-AGAGCCTGCAGCTTC TCAGA-3'
GTn reverse primer	5'-ACAAAGTCTGGCCATAGGAC-3'
-413A/T SNP (rs2071746)	
-413A/T forward primer	5'-TGACATTTTAGGGAGCTGGAGACA-3'
-413A/T reverse primer	5'-AGGCGTCCCAGAAGGTTCCA-3'
-413A/T probe for A allele	5'-FAM-CCCACCAGGCTATTGCTCTGAGCA-Tamra-3'
-413A/T probe for T allele	5'VIC-CCCACCAGGCTTTTGCTCTGAGC-Tamra-3'

Abbreviations: HO-1 = heme oxygenase-1, SNP = single-nucleotide polymorphism.

4.10.4 HO-1 plasma concentration

Plasma HO-1 levels were measured in serial samples, using the StressXpress human HO-1 ELISA kit (Stressgen). The plasma samples were diluted 1:5 in sample diluent and assayed according to manufacturers' instructions. The HO-1 sandwich ELISA assay is specific for HO-1 and does not cross-react with other HO isoforms. The intra- and interassay coefficients of variation reported by the manufacturer are both < 10%.

4.10.5 Statistical analyses: clinical study

The data of the human study are shown as the median and interquartile range (IQR) for continuous variables or as absolute values and percentages. Differences between variables were considered significant at a P value of < 0.05. Statistical analyses were performed, using SPSS software (versions 12.0 or 16.0). The GraphROC for Windows software was used in receiver-operating characteristic (ROC) analysis (Kairisto and Poola 1995).

Differences in the continuous variables were compared with the nonparametric Mann-Whitney *U*-test or Kruskall-Wallis test. Comparison of the categorical variables was performed, using the chi-square test or Fisher's exact test if the number of cases in a sample were lower than five. Bivariate correlations were determined, using the nonparametric Spearman's correlation. Linear regression analysis was used to determine the variables having independent effects on day 1 HO-1 concentrations and multivariate forward logistic regression analysis was performed to identify the variables having independent predictive value for ICU and hospital mortalities. Log-transformed values were used for variables with nonnormal distribution in linear regression and logistic regression analyses. ROC curves were constructed to determine the discriminative

power of HO-1 plasma concentrations regarding ICU and hospital mortalities. The area under the curve (AUC) was calculated with 95% confidence interval (CI). The best predictive cutoff values maximizing the sum of sensitivity and specificity were defined, and sensitivity, specificity, and positive likelihood ratios with 95% CIs were calculated.

Hardy-Weinberg equilibrium was calculated with Pearson's correlation and Fisher's exact tests. Pairwise linkage disequilibrium (LD) measures were determined and haplotypes were constructed by the solid spine of the LD algorithm, using Haploview 4.0 software (Barrett et al. 2005). In addition to the marker-by-marker analysis, we evaluated the associations by haplotype-based analysis, which is considered more capable of detecting associations (Akey et al. 2001, Zhang et al. 2002). The Genetic Power Calculator was used for power calculations (Purcell et al. 2003). With a relative risk of 2.5 for the heterozygotes and 3 for minor allele homozygotes regarding hospital mortality, our sample had 83% power to detect association with the +99G/C polymorphism.

4.11 Ethical aspects

The local Ethical Committees for Animal Experiments approved the experimental studies. All the animal experiments conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, Bethesda, MD, USA (NIH Publication No. 85-23, revised 1996). The Ethics Committee of Medicine, the Ethics Committee of Surgery, and the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa approved the human study.

5 RESULTS

5.1 HO-1 expression is induced in infarcted rat hearts *in vivo* (I–III)

Previous studies have shown that HO-1 is induced in response to I/R, but less is known of expression of HO-1 during the recovery phase of MI. Therefore, cardiac HO-1 expression was thoroughly investigated in post-MI rat hearts. The expression of HO-1 was increased in the post-MI hearts, both at the mRNA and protein levels, as indicated by real-time RT-PCR, HO-1 ELISA, and immunohistochemical analysis. HO-1 expression was particularly increased in the infarct area and in the infarct border area, as shown in Table 11. The expression of HO-1 mRNA peaked at day 1 in the infarct border area and at day 3 in the infarct area. HO-1 mRNA levels remained high in the infarct and infarct border area at week 1 and decreased back to levels comparable with sham-operated hearts by week 4. The results were similar in both MI experiments (I vs. II and III). HO-1 protein expression peaked in the infarct area at day 3 and remained high at week 1. The HO-1 protein levels decreased slowly thereafter, but unlike the HO-1 mRNA, the HO-1 protein levels still remained 5-fold higher in the infarct area and 2-fold higher in the infarct border area than in the sham-operated hearts at week 4. In addition, a transient increase in both HO-1 mRNA and protein levels was found in the remote area at week 1 in both MI experiments.

Table 11. HO-1 expression in infarcted rat hearts.

	HO-1 mRNA copies ($x10^4$) per ng total RNA Study I	HO-1 mRNA copies ($x10^4$) per ng total RNA Studies II and III	HO-1 protein ng per mg total protein Studies II and III
1 day			
Border area	19.0±3.2 *** †††	12.5±1.7 ** ††	1.8±0.26 * †
Remote area	6.6±0.7	2.1±0.2	0.8±0.09
Sham	4.9±0.6	2.0±0.2	0.7±0.08
3 days			
Infarct area		36.8±4.0 ** ††† ‡‡‡	7.5±0.69 *** ††† ‡‡‡
Border area		7.2±1.3 *	1.8±0.19 ** †††
Remote area		2.8±0.4	0.6±0.06
Sham		2.4±0.4	0.4±0.05
1 week			
Infarct area		14.2±2.1 ** ††† ‡‡‡	6.8±1.01 *** ††† ‡‡‡
Border area	9.0±1.3 *** †††	5.8±0.7 ** ††	1.3±0.15 ** †
Remote area	2.8±0.3 *	3.0±0.3 **	0.8±0.12 *
Sham	1.8±0.3	1.4±0.1	0.4±0.04
4 weeks			
Infarct area		1.7±0.1	2.0±0.16 ** †† ‡‡
Border area	2.4±0.3	2.1±0.2	0.8±0.07 ** †
Remote area	2.5±0.2	1.9±0.1	0.5±0.04
Sham	2.3±0.3	1.2±0.1	0.4±0.03

Values are mean ± SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. sham-operated hearts at the same time point. † P < 0.05, †† P < 0.01 and ††† P < 0.001 vs. remote area at the same time point. ‡‡ P < 0.01 and ‡‡‡ P < 0.001 vs. infarct border area at the same time point. HO-1 = heme oxygenase-1.

HO-1 protein was localized in the coronary artery walls throughout the myocardium, both in infarcted and sham-operated hearts (Fig. 5 A, B), and in the small arterioles of the infarct border area (Fig. 5 C). HO-1+ coronary arteries were found at all time points from day 1 to week 4, whereas HO-1+ small arterioles were observed only at week 1. HO-1 expression was also increased in the cardiomyocytes of the infarct border area during the first week after infarction (Fig. 5 C–E). HO-1 showed a distinct transverse and a less pronounced longitudinal striated pattern of staining in cardiomyocytes compatible with the localization of HO-1 protein in the sarcoplasmic reticulum (SR) (Fig. 5 E, F and Study I, Fig. 4). In addition, HO-1 protein was found at the intercalated discs of the cardiomyocytes (Fig. 5 D and Study I, Fig. 4). Increased expression of HO-1 was also found in the infarct area, specifically in fibroblastlike cells (Fig. 5 G) and in monocytes/macrophages (Fig. 5 H, unpublished result). The time course of HO-1 expression in different cardiac cell types or structures is shown in Table 12. In the remote myocardium or in the sham-operated hearts, HO-1 was found only in the coronary artery walls, not in cardiomyocytes or fibroblasts.

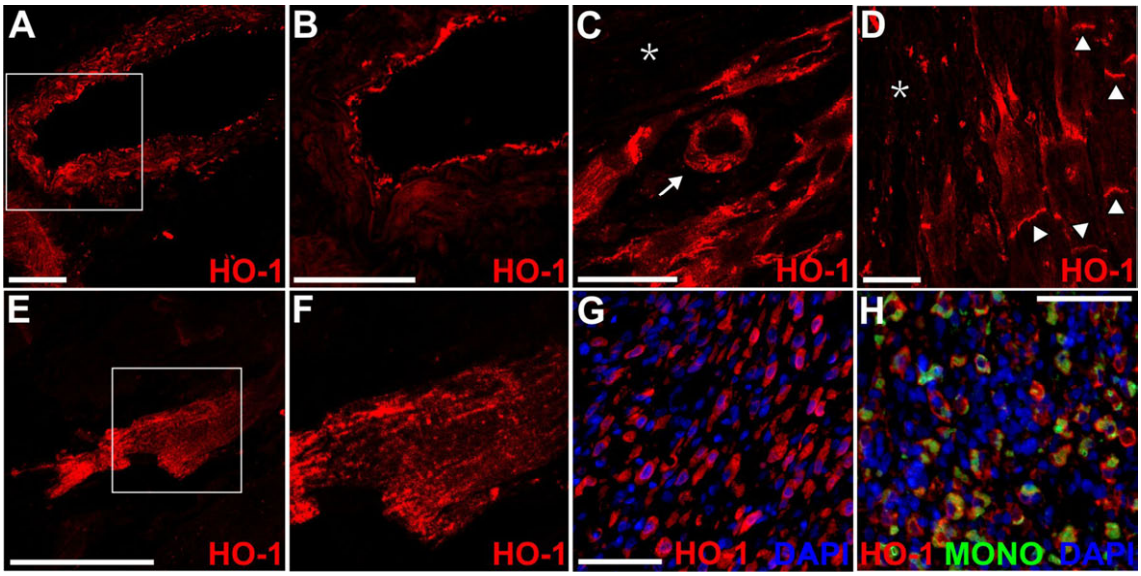

Figure 5. Localization of heme oxygenase-1 (HO-1) protein in the infarcted rat hearts. A) Representative image of HO-1 protein in the coronary artery wall. A higher magnification in panel B) shows the strongest HO-1 immunoreactivity in the endothelium of the coronary artery. C) HO-1+ arteriole (arrow) and cardiomyocytes in the infarct border area. D) HO-1+ cardiomyocytes in the infarct border area with the strongest HO-1 immunoreactivity in intercalated discs (arrowheads). E) HO-1+ cardiomyocyte in the infarct border area, showing both transverse and longitudinal striated patterns of staining. A higher magnification in panel F) shows the intracellular distribution of HO-1 in the cardiomyocyte. G) HO-1+ fibroblastlike cells in the infarct area at week 1 post-MI. H) Highly increased HO-1 expression in infiltrating monocytes/ macrophages in the infarct area at day 3 post-MI. * Infarct area. Scale bar = 50 μm.

Table 12. Time course of heme oxygenase-1 expression in cardiac cells and structures after infarction.

	Day 1	Day 3	Week 1	Week 4	Sham
Coronary arteries	X	X	X	X	X
Arterioles			X		
Cardiomyocytes	X	X	X		
Fibroblastlike cells		X	X	X	
Monocytes/macrophages		X	X		

X = Heme oxygenase-1 immunoreactivity detected by immunohistochemical analysis.

5.2 HO-1 and CO protect the heart (II–IV)

5.2.1 Infarct size (II, III)

MI was produced in rats by LAD ligation. The effects of pretreatments on infarct size after LAD ligation were assessed by planimetry. As expected, we found no statistically significant differences between HO-1-induced, CO donor-pretreated, and control MI groups in infarct size, but there was a trend for smaller infarcts to occur in the HO-1-induced group at weeks 1 and 4 after infarction (HO-1 29.4 ± 3.7% vs. control MI 41.6 ± 4.0%, P = 0.07 and HO-1 26.2 ± 4.0% vs. control MI 35.8 ± 7.4%, P = 0.24, respectively) (Table 13).

5.2.2 HO-1 and CO inhibit cardiomyocyte apoptosis (III)

The effects of HO-1 induction and CO donor pretreatment on cardiomyocyte apoptosis were analyzed in post-MI hearts, since HO-1 is known to inhibit apoptosis predominantly via CO in different organs and cells. In the present study, the percentage of TUNEL+ apoptotic cardiomyocytes in sham-operated hearts was low (0.016 ± 0.0034%) and no differences were found between groups. In the infarcted hearts, cardiomyocyte apoptosis peaked in the infarct border area at day 1 and deecreased slowly thereafter (Table 13). In the remote myocardium, cardiomyocyte apoptosis peaked at day 1 and week 4 (Table 13). HO-1 induction significantly reduced cardiomyocyte apoptosis in the infarct border area at day 3, week 1, and week 4 and in the remote myocardium at weeks 1 and 4 after infarction (Table 13). Pretreatment with CO resulted in similar reductions of cardiomyocyte apoptosis in the infarct border area at day 3 and week 1 and in the remote myocardium at week 1 after MI (Table 13).

5.2.3 HO-1 and CO modulate cardiomyocyte DNA repair (III)

Immunohistochemical staining of PCNA was performed to evaluate whether HO-1 and CO modulate the repair of cardiomyocyte DNA in post-MI rat hearts. PCNA is a marker of both DNA synthesis and repair and so is expressed in proliferating cells and in cells undergoing DNA repair. In the sham-operated hearts, the percentage of PCNA+ cardiomyocytes was low (0.0077 ± 0.0024%) and no differences were found between groups. In the infarcted hearts, HO-1 induction significantly increased the percentage of PCNA+ cardiomyocytes in the infarct border area at day 1 (3-fold, P = 0.030) and day 3 (2.3-fold, P = 0.009) compared with control MI hearts, whereas at weeks 1 and 4 a trend for reduced number of PCNA+ cardiomyocytes was found (HO-1 0.43 ± 0.11% vs. control MI 0.78 ± 0.13%, P = 0.072, and HO-1 0.69 ± 0.18% vs. control MI 1.37 ± 0.37%, P = 0.074, respectively) (Table 13). Unlike HO-1, CO donor pretreatment affected only the number of PCNA+ cardiomyocytes in the infarct border at week 4 (Table 13). In the remote myocardium, the percentage of PCNA+ cardiomyocytes was stable in both HO-1-induced and CO donor-pretreated hearts throughout the study (HO-1 0.023 ± 0.005% and CO 0.028 ± 0.009%) and in control hearts from day 1 to week 1 (0.025 ± 0.008%). In contrast, the percentage of PCNA+ cardiomyocytes in the remote myocardium was about 3-fold higher in control MI hearts than in HO-1-induced and CO donor-pretreated hearts at week 4 (Table 13).

Table 13. Effects of HO-1 induction and CO donor pretreatment on infarct size and percentage of apoptotic and PCNA+ cardiomyocytes.

	Infarct size (%)	Apoptotic cardiomyocytes (%)	PCNA+ cardiomyocytes (%)
1 day HO-1	40.8±9.7		
Border area		2.60±0.79	4.78±0.67 *
Remote area		0.089±0.014	0.024±0.005
1 day CO	36.1±5.5		
Border area		2.64±0.59	1.64±0.40
Remote area		0.091±0.024	0.042±0.013
1 day control	38.4±8.3		
Border area		2.91±0.95	1.53±0.20
Remote area		0.087±0.028	0.031±0.010
3 days HO-1	36.5±5.5		
Border area		0.59±0.037 **	4.91±0.59 **
Remote area		0.080±0.007	0.035±0.008
3 days CO	29.4±3.4		
Border area		0.56±0.062 *	2.90±0.53
Remote area		0.079±0.009	0.016±0.005
3 days control	36.0±5.6		
Border area		0.91±0.094	2.10±0.29
Remote area		0.092±0.008	0.022±0.009
1 week HO-1	29.4±3.7		
Border area		0.16±0.011 **	0.43±0.085 ‡
Remote area		0.024±0.005 †	0.020±0.005
1 week CO	35.0±5.9		
Border area		0.18±0.022 *	1.09±0.15
Remote area		0.025±0.004 †	0.028±0.010
1 week control	41.6±4.0		
Border area		0.31±0.054	0.78±0.13
Remote area		0.054±0.011	0.021±0.004
4 weeks HO-1	26.2±4.0		
Border area		0.18±0.029 **	0.69±0.15
Remote area		0.056±0.006†	0.014±0.003 ††
4 weeks CO	37.0±6.2		
Border area		0.28±0.035	2.16±0.28 * ##
Remote area		0.070±0.012	0.026±0.007 †
4 weeks control	35.8±7.4		
Border area		0.31±0.031	1.37±0.31
Remote area		0.093±0.010	0.071±0.010

Values are mean ± SEM. * P < 0.05 and ** P < 0.01 vs. border area of control MI hearts at the same time point. † P < 0.05 vs. remote area of control MI hearts at the same time point. ‡ P < 0.05 vs. border area of CO pretreated hearts at the same time point. ## P < 0.01 vs. border area of HO-1 induced hearts at the same time point. Unpublished results of apoptotic and PCNA+ cardiomyocytes in CO pretreated groups are shown in the table. Abbreviations: CO = carbon monoxide, HO-1 = heme oxygenase-1, MI = myocardial infarction, PCNA = proliferating cell nuclear antigen.

5.2.4 HO-1 improves postischemic cardiac function (IV)

The effect of HO-1 on postischemic cardiac function was studied in isolated I/R rat hearts. HO-1 induction by hemin significantly enhanced the recovery of postischemic cardiac function of isolated perfused rat hearts. HO-1 induction improved AF, CF, LVDP and -dp/dt, and decreased LVEDP compared with the nonpretreated I/R group (Table 14 and study IV, Table 1). HO-1 induction also significantly reduced I/R-induced VF to 12.5% compared with 100% in the control group (Table 14 and study IV, Table 1). The HO enzyme inhibitor ZnPPIX did not affect myocardial function compared with the control I/R group (study IV, Table I).

Table 14. Effect of HO-1 induction on postischemic cardiac function

Group	AF (ml/min)	LVDP (kPa)	-dp/dt (kPa/s)	LVEDP (kPa)	CF (ml/min)	VF (%)
1a Control I/R	9.1±2.3	11.8±0.5	316±13	1.77±0.05	15.9±0.7	100
2a HO-1 induced I/R	27.3±1.4 ***	14.3±0.3 **	401±16 **	1.23±0.06 ***	19.5±0.9 **	12.5 **

Abbreviations: HO-1 = heme oxygenase-1, I/R = ischemia/reperfusion, AF = aortic flow, LVDP = left ventricular developed pressure, -dp/dt = negative first derivative of LV pressure, LVEDP = left ventricular end-diastolic pressure, and CF = coronary flow during the reperfusion. VF = incidence of ventricular fibrillation during the reperfusion. Data are presented as mean ± SEM or as percentages (VF). ** $P < 0.01$ and *** $P < 0.001$ vs. control I/R group.

Reduced cardiac cGMP levels and abnormalities in the expression, distribution and phosphorylation of Cx43 are known to increase susceptibility to arrhythmias. Cardiac cGMP levels and Cx43 protein levels were measured to evaluate the role of cGMP and Cx43 in mediating the improved recovery of postischemic cardiac function and reduced VF by HO-1. However, no differences were found between groups in cardiac cGMP levels or in the levels of 44–46-kDa phosphorylated forms of Cx43. In contrast, the levels of nonphosphorylated Cx43 (at S368) were significantly higher in the HO-1-induced I/R hearts than in the nonpretreated I/R group and ZnPPIX-pretreated I/R group (Table 15 and study IV, Fig. 5B). In addition, the cardiac total Cx43 content (phosphorylated plus nonphosphorylated Cx43) was significantly higher in the HO-1-induced I/R group than in the nonpretreated I/R group and ZnPPIX-pretreated I/R group (Table 15 and study IV, Fig. 5C), and comparable to the HO-1-induced, aerobically perfused, time-matched control group (IV, Fig. 5C). The significantly increased nonphosphorylated Cx43 in HO-1-induced I/R hearts was localized, both in the intercalated discs and lateral plasma membrane, whereas in aerobically perfused hearts Cx43 was mainly in its phosphorylated form and localized principally in the intercalated discs.

Table 15. Effect of HO-1 induction on cardiac cGMP and Cx43 levels

Group	cGMP (fmol/mg tissue)	Phosphorylated Cx43	Nonphosphorylated Cx43	Total Cx43
1a Control I/R	1.38±0.24	1.70±0.44	1.49±0.17	1.52±0.19
2a HO-1-induced I/R	1.43±0.28	2.33±0.40	2.41±0.21 ** ††	2.44±0.20 * †
3a ZnPPIX I/R	1.42±0.20	1.59±0.21	1.37±0.12	1.42±0.12

Values are mean ± SEM. Cx43 results are reported as fold change vs. baseline. * $P < 0.05$ and ** $P < 0.01$ vs. control I/R group. † $P < 0.05$ and †† $P < 0.01$ vs. ZnPP I/R group. Abbreviations: HO-1 = heme oxygenase-1, cGMP = guanosine 3'5'-cyclic monophosphate, Cx43 = connexin 43, I/R = ischemia/ reperfusion, ZnPPIX = zinc protoporphyrin IX.

5.3 HO-1 and CO promote neovascularization and myocardial regeneration in infarcted rat hearts (II)

5.3.1 Differential effects of HO-1 and CO on capillary and vascular density

Immunostaining of vWF and α-SMA was performed to assess the effects of HO-1 induction and CO donor pretreatment on capillary and vascular densities at weeks 1 and 4 after infarction. The numbers of vWF+ capillaries and α-SMA+ vessels in infarcted and sham-operated hearts are shown in Table 16. The number of capillaries in the infarct area was increased in all groups at week 4 compared with week 1 (Table 16 and study II, Fig. 5B). Furthermore, HO-1 induction significantly increased the capillary density in the infarct area at week 4 compared with the control hearts, and a trend for increased capillary density by HO-1 induction was already found at week 1 (Table 16 and study II, Fig. 5B). In contrast, CO pretreatment did not affect capillary density compared with the control MI hearts. In addition, capillary density did not differ between groups in the infarct border area, remote myocardium, or sham-operated hearts (Table 16).

Table 16. Effects of HO-1 and CO on capillary and vascular densities in infarcted rat hearts.

	Capillary density	Vascular density	Intermediate arteries	Large arteries
Infarct area week 1				
HO-1	58±6 †	252±43	6.1±0.5 * ††	3.2±0.3 * ††
CO	38±4	254±38	3.2±0.6	1.9±0.2
Control	43±4	259±46	4.5±0.2	2.4±0.5
Infarct area week 4				
HO-1	161±29 ** † ‡‡	446±26 * ‡	13.3±0.7 ** ‡‡	6.5±0.4 * ‡‡
CO	90±24 ‡	395±24 * ‡	12.7±0.9 * ‡‡‡	7.1±0.6 ** ‡‡‡
Control	70±10 ‡	306±25 ‡	9.7±0.5 ‡‡	4.8±0.4 ‡‡
Border area week 1				
HO-1	1324±95	354±54		
CO	1016±69	282±52		
Control	1144±63	301±58		
Border area week 4				
HO-1	976±52	182±26		
CO	918±96	182±30		
Control	930±45	185±26		
Remote area week 1				
HO-1	1876±60	105±10		
CO	1650±37	104±14		
Control	1767±70	116±14		
Remote area week 4				
HO-1	1533±85	148±7		
CO	1457±127	130±7		
Control	1404±53	145±12		
Sham week 4				
HO-1	1799±169	146±23		
CO	1523±257	152±18		
Control	1729±146	152±31		

Values are mean ± SEM and results are reported as vessel count per mm^2. * $P < 0.05$ and ** $P < 0.01$ vs. control group at the same time point. † $P < 0.05$ and †† $P < 0.01$ vs. CO-pretreated group at the same time point. ‡ $P < 0.05$, ‡‡ $P < 0.01$ and ‡‡‡ $P < 0.001$ vs. week 1. Unpublished results of capillary and vascular densities in the infarct border and remote areas and in sham-operated hearts are included in the table. Abbreviations: HO-1 = heme oxygenase-1, CO = carbon monoxide.

Both HO-1 induction and CO donor pretreatment significantly increased the vascular density in the infarct area at week 4 compared with the control MI hearts and with the vascular densities at week 1 (Table 16 and study II, Fig. 5A). In addition to the total number of α-SMA+ vessels, the numbers of intermediate (25–65 μm diameter) and large (> 65-μm diameter) arteries were also counted from the infarct areas. HO-1 induction increased the number of intermediate and large arteries in the infarct area compared with the control MI hearts already at week 1 and further increase was seen at week 4 (Table 16 and study II, Fig. 5C). Unlike HO-1 induction, CO pretreatment increased intermediate and large arteries only at week 4, but the fold increase at week 4 compared with week 1 was greatest in CO-pretreated hearts: increase in intermediate arteries was 4-fold and increase in large arteries 3.7-fold (Table 16 and study II, Fig. 5C). In HO-1-induced and control MI hearts, the increase in intermediate and large arteries from week 1 to week 4 was about 2-fold. Similar to capillary density, vascular density did not differ between groups in the infarct border area, remote myocardium, or sham-operated hearts.

5.3.2 CO activates c-kit+ stem/progenitor cells and promotes differentiation of c-kit+ cells into vascular smooth muscle cells and cardiomyocytes

Immunohistochemical analysis of c-kit was performed to assess whether HO-1 and CO promote activation of cardiac stem cells in the infarcted rat hearts. In comparison to control MI hearts, CO donor pretreatment significantly increased the number of c-kit+ stem/progenitor cells in the infarct area at weeks 1 and 4 after infarction (2.6-fold, P = 0.038 and 89-fold, P = 0.000, respectively), whereas only occasional c-kit+ cells were found at days 1 and 3 and no c-kit+ cells were found in sham-operated hearts (study II, Fig. 2). Immunohistochemical staining was used to further characterize these CO-induced c-kit+ cells, in which 3% of the cells were proliferating cells (c-kit+/Ki67+), 10% of the c-kit+ cells expressed the early cardiac/cardiomyocyte transcription factor Nkx2.5 (c-kit+/Nkx2.5+), 41% expressed the smooth muscle transcription factor GATA6 (c-kit+/GATA6+), and 1% were positive for the endothelial lineage marker Ets-1 (c-kit+/Ets-1+) (study II, Fig. 3). Immunostaining was also used to evaluate the origin of the c-kit+ cells and we found that these c-kit+ cells were negative for the hematopoietic stem cell marker CD34 (study II, Fig. 2). The c-kit+ cells are also negative for Sca-1 and nestin (data not shown), whereas some c-kit+ cells were positive for T-box18 (Tbx18), a marker for epicardium-derived stem/progenitor cells (EDPCs) (Fig. 6) (Lakkisto et al., unpublished results). Unlike CO donor pretreatment, HO-1 induction increased the number of c-kit+ cells only at week 4 after infarction (11-fold, P = 0.001) (study II, Fig. 2). A few occasional c-kit+ cells were found at earlier time points and as in CO pretreatment, no c-kit+ cells were found in HO-1-induced sham-operated hearts.

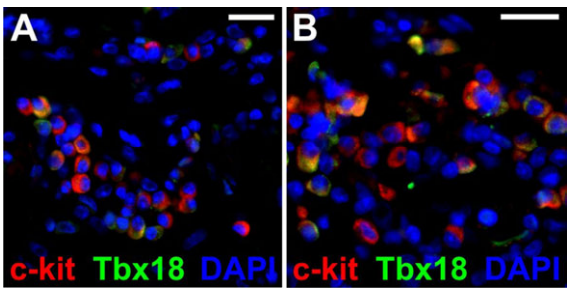

Figure 6. Stem/progenitor cells in the infarct area. A-B) Representative images of c-kit^{pos}/Tbx18^{pos} stem/progenitor cells and c-kit^{pos}/Tbx18^{neg} stem/progenitor cells in the infarct areas of carbon monoxide (CO) donor-pretreated hearts at week 4. Scale bar = 20 μm.

5.3.3 HO-1 and CO modulate expression of angiogenic factors

HIF-1α, SDF-1α, VEGF-A, and VEGF-B are key factors promoting neovascularization and cardiac regeneration. Cardiac levels of these factors were measured to evaluate their role in HO-1- and CO-induced neovascularization and in the activation of c-kit+ stem/progenitor cells by CO. In comparison to control MI hearts, CO donor pretreatment increased HIF-1α protein levels in the infarct areas 2-fold at day 3 and week 1 (study II, Fig. 6) and VEGF-B protein levels 1.6-fold at week 4 (study II, Fig. 8). Cardiac SDF-1α protein levels were increased in all infarcted hearts compared with sham-operated hearts from day 3 to week 1 after MI (study II, Fig. 7). In HO-1-induced and CO donor-pretreated hearts, the SDF-1α levels remained high at week 4, while in the control hearts the SDF-1α levels decreased to the levels of sham-operated hearts. In comparison to the control MI hearts SDF-1α protein levels were 2-fold higher in the infarct areas of the HO-1-induced and CO-pretreated hearts at week 4 (study II, Fig. 7). In contrast to CO, HO-1 induction did not significantly affect the HIF-1α or VEGF-B protein levels. No significant differences were found between the groups in the VEGF-A protein levels.

5.4 HO-1 and CO modulate proliferation of cardiac cells in infarcted rat hearts *in vivo* (II, III)

5.4.1 Proliferation of cells in the infarcted rat hearts (III)

HO-1 regulates proliferation of several cell types *in vitro*. Immunohistochemical analysis of Ki67 and DAPI was performed to assess the number of all proliferating cells in comparison to the total cell count and the effects of HO-1 and CO on proliferation of cells in the infarcted hearts *in vivo*. In sham-operated hearts, the total cell number was 2648 ± 81 cells per mm^2 and did not differ by pretreatment or time point. In the infarcted hearts, the total cell count was lower in the infarct areas of HO-1-induced hearts than in the control MI hearts at day 3 (5825 ± 169 vs. 7110 ± 282 cells per mm^2, P = 0.007, study III, Fig. 4 and Supplemental Table 2). HO-1 induction also reduced the number of proliferating cells in the infarct area at day 3 and in the infarct border area at week 1, and a trend for reduced proliferation was found in the infarct area at week 1 (5.8 ± 0.8% vs. 8.2 ± 0.7% Ki67+ cells, P = 0.068; Table 17 and study III, Figures 4 and 5 and Supplemental Table 2). In contrast to HO-1 induction, no significant changes were observed in proliferation of cells in CO donor-pretreated hearts, although a trend for reduced proliferation of cells was seen in the infarct area at week 1 (CO 6.0 ± 0.4% vs. control MI 8.2 ± 0.7% Ki67+ cells, P = 0.073; Table 17).

5.4.2 Proliferation of cardiomyocytes in the infarcted rat hearts (II, III)

HO-1 inhibits proliferation of VSMCs and airway SMCs, but the effects of HO-1 and CO on proliferation of cardiomyocytes have not been studied earlier. The proliferation of cardiomyocytes was assessed by immunostaining of Ki67 and cardiac myosin. In sham-operated hearts the percentage of proliferating Ki67+ cardiomyocytes was very low (0.0024 ± 0.0005%) and no differences were found between the groups. In the infarcted hearts, HO-1 induction significantly increased the percentage of Ki67+ cardiomyocytes in the infarct border area at days 1 and 3, while reducing the number of Ki67+ cardiomyocytes by week 1 after MI (Table 17 and study III, Fig. 2). HO-1 also reduced the number of Ki67+ cardiomyocytes in the remote myocardium at day 3 and weeks 1 and 4 (Table 17 and study III, Fig. 2). The effects of CO donor pretreatment differed from those of HO-1 induction, because this pretreatment increased

Table 17. Proliferation of cardiac cells in infarcted rat hearts

Group	Ki67+ cells infarct (%)	Ki67+ cells border (%)	Ki67+ cells remote (%)	Ki67+ cardiomyocytes border (%)	Ki67+ cardiomyocytes remote (%)	Ki67+ fibroblasts infarct (%)	Ki67+ fibroblasts border (%)	Ki67+ fibroblasts remote (%)
1 day								
HO-1	8.7±2.5	2.8±0.2	1.6±0.1	0.37±0.06 ** †	0.0034±0.001	17.7±3.2	6.9±0.7	5.1±0.8
CO	7.0±0.8	2.9±0.5	1.7±0.3	0.13±0.03	0.0044±0.001	16.2±0.7	7.2±2.3	3.8±0.9
Control	8.7±1.8	3.2±0.2	1.6±0.2	0.12±0.02	0.0029±0.0008	12.8±3.3	6.1±0.6	2.9±0.4
3 days								
HO-1	27.9±2.0 * †	9.7±1.3	1.1±0.3	0.46±0.05 ** †	0.0019±0.0001 ***	23.1±4.9	16.7±1.6	2.7±0.3
CO	38.3±3.4	9.8±1.8	1.3±0.3	0.30±0.02	0.0026±0.0003 **	31.6±2.8 #	22.4±3.1	3.0±0.6
Control	35.4±1.5	8.5±0.9	1.1±0.1	0.24±0.04	0.0053±0.0004	27.5±2.1	15.4±1.8	2.3±0.4
1 week								
HO-1	5.8±0.8	2.3±0.3 **	1.0±0.2	0.04±0.010 * †	0.0045±0.0007 ** †	6.0±0.6	3.8±0.9 *	2.4±0.6
CO	6.0±0.4	3.4±0.7	1.7±0.6	0.15±0.034	0.0092±0.0010 *	4.9±0.6	6.0±1.3	4.3±1.2
Control	8.2±0.7	5.3±0.5	2.0±0.6	0.13±0.020	0.0151±0.0012	6.4±0.6	8.6±1.4	5.9±1.7
4 weeks								
HO-1	2.2±0.3 †	2.7±0.3	1.8±0.2	0.080±0.012	0.0084±0.0010 ** †	2.3±0.3 †	4.1±0.5	3.8±0.5
CO	3.4±0.3	2.7±0.3	2.4±0.3	0.153±0.013 ** #	0.0129±0.0017	3.7±0.4	4.5±0.9	4.0±0.7
Control	2.8±0.4	2.0±0.2	2.1±0.2	0.086±0.008	0.0142±0.0009	3.8±0.8	4.5±0.6	3.9±0.5

Values are mean ± SEM and the results are reported as the percentage of Ki67+ cells of the total cell count (Ki67+ cells), the total cardiomyocyte count or the total fibroblast count. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control MI group at the same time point. † $P < 0.05$ vs. CO-pretreated group at the same time point. # $P < 0.05$ vs. HO-1-induced group at the same time point. Unpublished results for CO-pretreated groups are included in the table. Abbreviations: HO-1 = heme oxygenase-1, CO = carbon monoxide.

proliferation of cardiomyocytes only in the infarct border area at 4 weeks post-MI (Table 17 and study II, Fig. 4). However, CO donor pretreatment also reduced proliferation of cardiomyocytes in the remote myocardium at day 3 and week 1 (Table 17).

5.4.3 Proliferation of fibroblasts in the infarcted rat hearts (III)

Fibroblasts have a key role in post-MI ventricular remodeling. The number and proliferation of fibroblasts was assessed by immunohistochemical analysis of Ki67 and vimentin to evaluate whether HO-1 and CO would inhibit proliferation of fibroblasts in the infarcted hearts *in vivo*. The fibroblast counts in infarcted rat hearts are shown in study III, Supplemental Table 3. HO-1 induction reduced the number of vimentin+ fibroblastlike cells in the infarct area (3535 ± 190 vs. 4632 ± 317 fibroblasts per mm^2, P = 0.015) while slightly increasing the number of vimentin+ cells in the infarct border area at day 3 (1195 ± 58 vs. 1004 ± 32 fibroblasts per mm^2, P = 0.018) (study III, Figures 4 and 5 and Supplemental Table 3). HO-1 induction also decreased proliferation of fibroblasts in the infarct border area at week 1 and a trend for decreased proliferation of fibroblasts was found in the infarct area at week 4 (2.3 ± 0.3% vs. 3.8 ± 0.8% Ki67+/vimentin+ cells, P = 0.074; Table 17 and study III, Figures 4 and 5 and Supplemental Table 3). In comparison to control MI hearts, no significant changes were found in proliferation of fibroblasts in CO-pretreated hearts (Table 17).

5.5 Effects of HO-1 and CO on fibrosis and expression of extracellular matrix components in infarcted rat hearts (III)

5.5.1 Interstitial and perivascular fibrosis and cardiomyocyte cross-sectional area

Post-MI adverse ventricular remodeling is characterized by increased cardiac fibrosis and cardiomyocyte hypertrophy. The effects of HO-1 and CO on interstitial and perivascular fibroses were determined from the infarcted and sham-operated hearts at week 4 by picrosirius red staining. HO-1 induction decreased the extent of perivascular fibrosis in both infarcted hearts (1.29 ± 0.06 vs. 1.74 ± 0.13, P = 0.006) and sham operated hearts (1.14 ± 0.05 vs. 1.57 ± 0.08, P = 0.002), whereas CO donor pretreatment did not significantly affect perivascular fibrosis (Fig. 7). There were no significant differences between the groups in interstitial fibrosis (Fig. 7) or in the cardiomyocyte cross-sectional area (HO-1 and control groups shown in study III, Fig. 6).

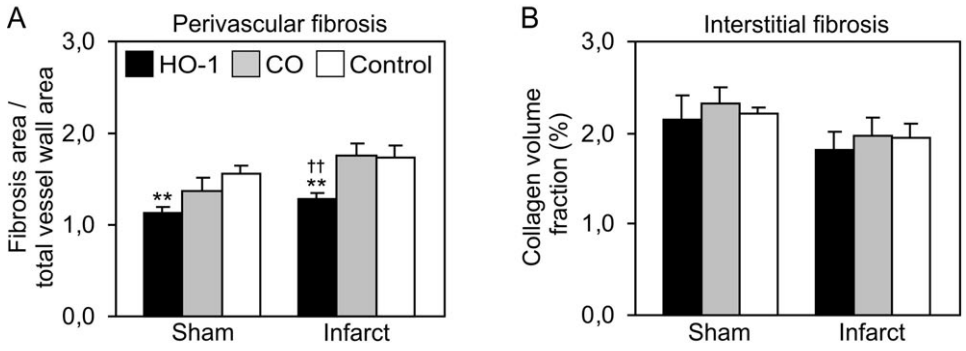

Figure 7. Interstitial and perivascular fibrosis. A) Ratio of perivascular fibrosis. B) The percentage of interstitial fibrosis. Values are mean ± SEM. ** P < 0.01 vs. control infarct or control sham group. †† P < 0.01 vs. CO-pretreated group. Unpublished results for CO-pretreated groups are included in the figure. Abbreviations: HO-1 = heme oxygenase-1, CO = carbon monoxide.

Table 18. Expression of extracellular matrix components.

Group	Coll1a1 mRNA Infarct	Coll1a1 mRNA Remote	Coll3a1 mRNA Infarct	Coll3a1 mRNA Remote	Fibronectin mRNA Infarct	Procollagen I protein Infarct	Procollagen I protein Remote	Vimentin protein Infarct	Vimentin protein Remote
3 days									
HO-1	237±10 †	16.7±2.0	54.4±2.8	9.5±1.1	14.1±1.6	0.65±0.17	1.28±0.28	1.36±0.10	0.84±0.27
CO	306±20	13.2±2.8	58.0±3.5	7.2±1.4	14.2±0.7	0.80±0.13	1.46±0.37	1.27±0.13	1.33±0.18
Control	263±27	24.8±5.8	57.9±2.9	9.8±1.8	14.4±2.1	0.90±0.33	2.44±0.75	1.41±0.23	1.08±0.20
1 week									
HO-1	224±34 * †	9.0±1.9 ††	84.9±7.5 †	9.4±1.7	17.1±3.2	1.03±0.05 * †	0.58±0.19 ††	0.70±0.25	0.83±0.35
CO	428±53	37.9±7.7	119.7±6.4	14.1±2.7	17.8±1.5	1.48±0.05	1.66±0.22	1.09±0.14	1.57±0.41
Control	362±26	31.4±9.7	100.2±4.7	10.6±2.4	14.8±1.5	1.49±0.13	1.73±0.63	0.96±0.12	1.44±0.60
4 weeks									
HO-1	49.2±9.4	13.1±1.9	21.1±2.4	5.5±0.8	3.0±0.4	1.05±0.12	0.96±0.09	0.72±0.10 *	0.58±0.11
CO	106.5±16.3 * #	15.4±1.8	40.7±4.5 ##	9.2±1.4 *	5.1±0.4 ** #	1.44±0.07	1.18±0.09	0.92±0.06	0.82±0.13
Control	54.8±8.8	11.8±1.8	24.3±4.7	5.1±0.7	2.3±0.5	1.40±0.28	1.34±0.22	1.42±0.17	1.05±0.22

Values are mean ± SEM. The mRNA data are reported as mRNA copies ($x10^4$) per ng of total RNA. Protein data are reported as a ratio of procollagen I or vimentin per actin. * $P < 0.05$ and ** $P < 0.01$ vs. control MI group at the same time point. † $P < 0.05$ and †† $P < 0.01$ vs. CO-pretreated group at the same time point. # $P < 0.05$ and ## $P < 0.01$ vs. HO-1-induced group at the same time point. Unpublished results for CO-pretreated groups are included in the table. Abbreviations: Coll1a1 = procollagen type I alpha 1, Coll3a1 = procollagen type III alpha 1, CO = carbon monoxide, HO-1 = heme oxygenase-1.

5.5.2 Expression of extracellular matrix components

Collagens type I and III, and fibronectin are major components of extracellular matrix. The effects of HO-1 and CO on procollagen type I (Coll1a1) and type III (Coll3a1), and fibronectin mRNA levels were analyzed by real-time RT-PCR. HO-1 induction decreased Coll1a1 mRNA expression in the infarct area at week 1 and a strong trend for decreased Coll1a1 mRNA expression was found in the remote area at the same time point (HO-1 9.0 ± 1.9 vs. CO 37.9 ± 7.7, P = 0.001 and control MI 31.4 ± 9.7 P = 0.053, Table 18 and study III, Fig. 7). However, HO-1 did not significantly affect Coll3a1 or fibronectin mRNA levels (Table 18). In contrast to HO-1, CO donor pretreatment increased Coll1a1, Coll3a1, and fibronectin mRNA levels in the infarcted hearts at week 4. Coll1a1 and fibronectin mRNAs were significantly increased by CO in the infarct area and Coll3a1 mRNA in the remote area (Table 18). In addition, a strong trend for increased Coll3a1 expression was found in the infarct area at weeks 1 and 4 (week 1: CO 119.7 ± 6.4 vs. control MI 100.2 ± 4.7, P = 0.053 and HO-1 84.9 ± 7.5, P = 0.010; week 4: CO 40.7 ± 4.5 vs. control MI 24.3 ± 4.7, P = 0.054 and HO-1 21.1 ± 2.4, P = 0.008, Table 18).

The effects of HO-1 and CO on procollagen type I and vimentin protein levels were measured by Western blotting. Vimentin protein expression was used as an indirect measure of fibroblast number. As in the Coll1a1 mRNA levels, HO-1 induction decreased the levels of procollagen type I protein in the infarct area at week 1 (Table 18 and study III, Fig. 7). In comparison to CO-pretreated hearts, HO-1 also decreased procollagen type I protein in the remote area at week 1 (Table 18). HO-1 also decreased vimentin protein in the infarct area at week 4 and a trend for decreased vimentin expression was observed in the remote area at week 4 (0.58 ± 0.11 vs. 1.05 ± 0.22, P = 0.093, Table 18 and study III, Fig. 7). Although CO pretreatment increased the mRNA levels of Coll1a1, Coll3a1, and fibronectin, no significant changes were found in the procollagen type I or vimentin protein levels by CO (Table 18).

5.5.3 Expression of CTGF, TGF-β1, and ANP

Connective tissue growth factor (CTGF) and transforming growth factor beta1 (TGF-β1) are key factors regulating cardiac fibrosis and ventricular remodelling while atrial natriuretic peptide (ANP) is a marker of heart failure. The mRNA levels of CTGF, TGF-β1 and ANP were measured by real-time RT-PCR. HO-1 induction decreased the expression of CTGF mRNA both in the infarct and remote areas at week 4 and a trend for lower CTGF levels was found in the infarct area at day 1 (6.9 ± 1.1 vs. 10.2 ± 0.9, P = 0.068) (Fig. 8 and study III, Fig. 8). In contrast, the CTGF mRNA levels were increased by HO-1 in the remote area at day 3 (Fig. 8 and study III, Fig. 8). The effects of CO pretreatment differed again from the effects of HO-1 induction, since CO significantly increased the expression of CTGF mRNA in the infarct area at week 4 (Fig. 8). No significant differences were found between the groups in CTGF mRNA levels in the infarct border area or sham-operated hearts.

Both HO-1 induction and CO donor pretreatment increased the TGF-β1 mRNA levels in the infarct area at day 3 (Fig. 8). Increased TGF-β1 mRNA levels were also found in the infarct areas of CO-pretreated hearts at week 4 (Fig. 8). No significant differences were found between the groups in TGF-β1 mRNA expression in the infarct border or remote areas or sham-operated hearts.

HO-1 induction decreased the levels of ANP mRNA in the infarct border area at day 3 and week 1 (2.0 ± 0.5 vs. 3.1 ± 0.3, P = 0.050) (Fig. 8 and study III, Fig. 8). Decreased ANP mRNA levels were also found in the infarct border areas of CO-pretreated hearts at week 1 (Fig. 8). No

significant differences were found between the groups in ANP mRNA levels in the infarct or remote areas or in the sham-operated hearts.

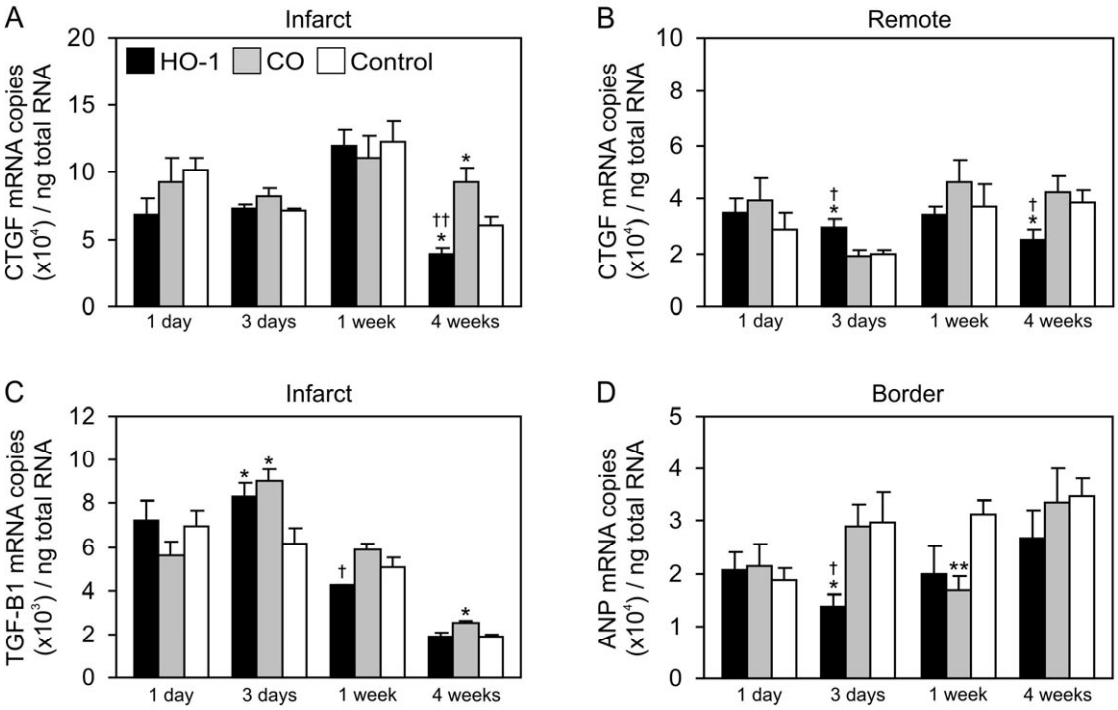

Figure 8. CTGF, TGF-β1 and ANP mRNA expression in the infarcted hearts. A) CTGF mRNA levels in the infarct area. B) CTGF mRNA levels in the remote area. C) TGF-β1 mRNA levels in the infarct area. D) ANP mRNA levels in the infarct border area. Values are mean ± SEM. * $P < 0.05$ and ** $P < 0.01$ vs. control MI group. † $P < 0.05$ and †† $P < 0.01$ vs. CO-pretreated group. Unpublished results for CO-pretreated groups are included in the figure. Abbreviations: ANP = atrial natriuretic peptide, CO = carbon monoxide, CTGF = connective tissue growth factor, HO-1 = heme oxygenase-1, TGF-β1 = transforming growth factor beta1.

5.6 Clinical study

5.6.1 *HO-1* polymorphisms

Different polymorphisms have been identified in the human *HO-1* gene. The GT_n repeat length polymorphism and the -413A/T SNP polymorphism are the most studied of *HO-1* polymorphisms. The allelic distribution of the *HO-1* GT_n repeat length polymorphism is shown in Fig. 9. The GT_n repeat length ranged from 18 to 40, and the most frequent repeat lengths were 30 (47.6%) and 23 (18.6%). Based on the trimodal allelic distribution, the GT_n repeat length was divided into short (S), medium (M), and long (L) alleles. The frequencies of the S, M and L alleles were 36%, 59%, and 5%, respectively. The *HO-1* SNPs (+99G/C and -413A/T) were in Hardy-Weinberg equilibrium. The frequencies of the +99G/C genotypes were: GC 10.4% (24/231) and GG 89.6% (207/231). The frequencies of the -413A/T genotypes were: AA 35.5% (82/231), AT 48.5% (112/231) and TT 16.0% (37/231). The allele frequencies did not differ between men and women.

Strong LD was observed between the polymorphisms studied. The D' measure of LD between the two SNPs (+99G/C and -413A/T) was 1.0, while the r^2 value that takes allele frequencies into

account was 0.08. The most common repeat length allele, GT(30), was in complete LD (D'=1) with the -413A and +99G alleles. The next most frequent repeat length alleles, GT(23) and GT(24), were in complete LD with the -413T and +99G alleles. In addition, the group of L alleles was in perfect LD with the +99C allele (both D' and r^2=1). The different haplotypes formed by the three polymorphisms studied and their relative frequencies are shown in study V, Table 2.

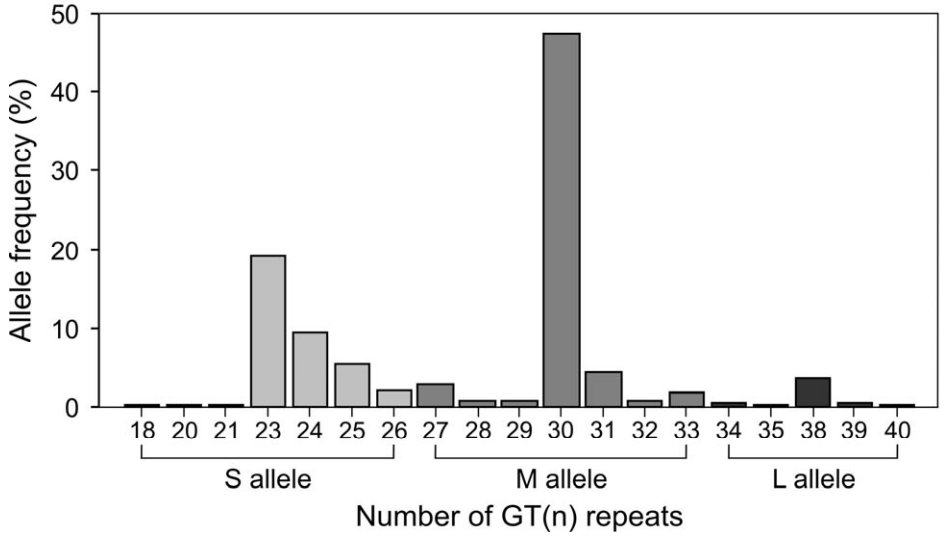

Figure 9. Allelic distribution of the *heme oxygenase-1 (HO-1)* GT_n repeat length polymorphism and classification of the GT_n repeats into S, M and L alleles.

5.6.2 HO-1 plasma concentrations

HO-1 plasma concentrations have been studied only in a few previous studies. The plasma samples of 58 healthy controls were used to establish the reference range for plasma HO-1. The plasma HO-1 reference range (95% CI) was 0.66–2.39 ng/ml, with a median of 1.5 ng/ml (IQR 1.13–1.77 ng/ml). The concentrations did not differ between men and women. The HO-1 plasma concentrations of the critically ill study patients were significantly higher than the reference values (Fig. 10 and study V, Fig. 2). The median day 1 plasma HO-1 concentration of the study patients was 6.9 ng/ml (IQR 5.1–9.9 ng/ml), the day 2 HO-1 6.6 ng/ml (IQR 4.9–9.3 ng/ml), the day 3-4 HO-1 6.4 ng/ml (IQR 4.9–8.1 ng/ml) and the maximum HO-1 concentration 7.3 ng/ml (IQR 5.6–10.2 ng/ml). The HO-1 levels were elevated in all critically ill patients regardless of the diagnosis (study V, Table 3). The HO-1 plasma levels were highest in patients with pancreatitis (9.0 ng/ml, IQR 7.7–14.5 ng/ml), sepsis (8.4 ng/ml, IQR 5.7–12.0 ng/ml), or respiratory tract infection (8.4 ng/ml, IQR 5.8–9.5 ng/ml). Slightly lower HO-1 plasma concentrations were measured from patients with heart failure (7.2 ng/ml, IQR 5.5–10.0 ng/ml), heart arrest (6.3 ng/ml, IQR 5.1–10.2 ng/ml), or acute MI (6.4 ng/ml, IQR 4.6–8.5 ng/ml). The HO-1 plasma levels were lowest in patients with cerebrovascular disorders (3.7 ng/ml, IQR 2.3–4.1 ng/ml) or intoxication (4.9 ng/ml, IQR 4.0–10.1 ng/ml). One patient was excluded from further analysis due to autoimmune hemolytic anemia and extremely high HO-1 concentration (407 ng/ml).

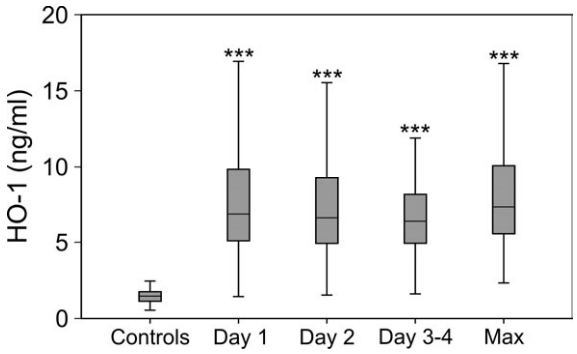

Figure 10. Heme oxygenase-1 (HO-1) plasma concentrations of controls and study patients at different time points. Values are shown as median (line) and interquartile range (IQR, boxes) and 5th and 95th percentiles (whiskers). *** P < 0.001 vs. control group. Unpublished data on maximum HO-1 levels is included in the figure.

5.6.3 Association of *HO-1* polymorphisms with HO-1 plasma concentrations, disease severity, and mortality

HO-1 polymorphisms have been shown to modulate HO-1 expression levels and associate with different diseases. In the present study, *HO-1* polymorphisms significantly affected the HO-1 plasma concentrations. The -413T/GT(L)/+99C haplotype was associated with significantly lower HO-1 concentrations at all time points compared with the other haplotypes (Fig. 11 and study V, Fig. 3). Of the 24 patients carrying the -413T/GT(L)/+99C haplotype, 14 had the day 1 plasma HO-1 value. The -413T allele was also associated with lower day 3-4 HO-1 plasma concentrations than the AA homozygotes (6.1 vs. 6.9 ng/ml, P = 0.021).

Patients with the -413T/GT(L)/+99C haplotype had less frequently multiple organ dysfunction (MOD) (SOFA score > 6) than patients with other haplotypes on day 1 in ICU (46% vs. 70%, P = 0.017). However, the *HO*-1 polymorphisms were not associated with ICU or hospital mortality or the severity of disease, as measured with the APACHE II and SAPS II scores.

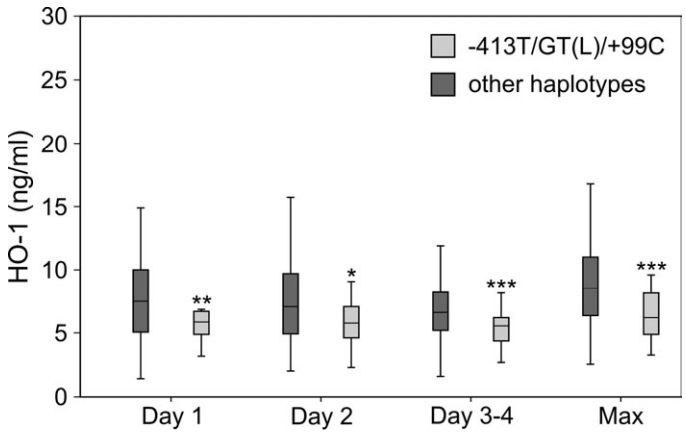

Figure 11. Patients with the *heme oxygenase-1 (HO-1)* -413T/GT(L)/+99C haplotype (n = 24) had significantly lower HO-1 concentrations compared to patients with other haplotypes (n = 207). Values are shown as median (line) and interquartile range (IQR, boxes) and 5th and 95th percentiles (whiskers). * P < 0.05, ** P < 0.01 and *** P < 0.001. Unpublished data on maximum HO-1 levels is included in the figure.

5.6.4 Association of HO-1 plasma concentrations with disease severity and mortality

HO-1 is a highly inducible stress responsive enzyme, and thereby a potential marker of disease severity and predictor of outcome in critically ill patients. Therefore, the association of HO-1 plasma concentrations with disease severity and mortality was investigated in this study. The day 1 and day 2 HO-1 concentrations correlated significantly with the day 1 and day 2 bilirubin levels (Table 19). In addition, the day 1 HO-1 concentration correlated significantly with CRP concentration, the disease severity scores APACHE II and SAPS II, the first-day SOFA score (especially the coagulation, liver, renal, and pulmonary scores) and the maximum SOFA score (Table 19). The day 1 HO-1 concentrations in the day 1 SOFA quartiles are shown in study V, Fig. 4. When organ failures were analyzed separately in the linear regression analysis, renal failure (P = 0.02) and ventilation failure (P = 0.02) were associated independently with the HO-1 values. Patients with MOD on the day 1 of intensive care had significantly higher HO-1 concentrations at all time points than patients with no MOD (day 1 HO-1 7.8 vs. 6.1 ng/ml, P = 0.015; day 2 6.9 vs. 5.6 ng/ml, P = 0.003; and day 3-4 6.5 vs. 5.6 ng/ml, P = 0.004).

Table 19. Correlation of HO-1 plasma concentrations with CRP, bilirubin and clinical scores.

Variable	r2	p
Day 1 CRP	0.18	0.02
Day 1 bilirubin	0.27	0.001
Day 2 bilirubin	0.26	0.001
APACHE	0.27	0.001
SAPS II	0.17	0.038
Day 1 SOFA	0.32	<0.001
Central nervous system SOFA	0.003	0.966
Cardiovascular SOFA	0.138	0.085
Coagulation SOFA	0.336	<0.001
Liver SOFA	0.373	<0.001
Renal SOFA	0.298	<0.001
Pulmonary SOFA	0.232	0.003
Max SOFA	0.34	<0.001

Abbreviations: APACHE II = acute physiology and chronic health evaluation II score, CRP = C-reactive protein, HO-1 = heme oxygenase-1, SAPS II = simplified acute physiology score II, SOFA = sequential organ failure assessment score.

Age did not correlate with HO-1 concentrations, but men had higher day 2, day 3-4, and maximum HO-1 concentrations than women (P < 0.001, P = 0.001 and P = 0.001, respectively), while their disease and organ failure scores did not differ from women's scores. In the linear regression analysis used to identify the variables independently associating with the day 1 HO-1 concentration, the day 1 CRP values, gender, carriage of the T/GT(L)/C haplotype, SAPS II score, and SOFA score were included. The day 1 SOFA score (P = 0.001) and T/GT(L)/C haplotype (P = 0.03) were associated independently with the day 1 HO-1 concentrations.

The ICU mortality rate was 7.4% (17/231) and the hospital mortality rate 19.5% (45/231). The ICU and hospital mortalities for those 160 patients (of 231) with the day 1 plasma HO-1 were 6% (10/160) and 21% (33/160). The patients with the day 1 plasma HO-1 did not differ from the patients without the plasma sample regarding hospital mortality, SAPS II, gender, SOFA, or the carriage of the T/GT(L)/C haplotype (data not shown), but they were slightly younger (median 57 vs. 61 years, P = 0.04). The day 1 HO-1 concentration and the maximum HO-1 concentration during the first 4 days in the ICU were significantly higher in patients who died in the ICU than

in ICU survivors (day 1 HO-1 11.4 vs. 6.5 ng/ml, P < 0.001 and maximum HO-1 9.9 vs. 7.2 ng/ml, P = 0.006, respectively). The maximum HO-1 concentration was also higher in hospital nonsurvivors than in survivors (8.4 vs. 7.1 ng/ml, P = 0.02), but the other HO-1 concentrations (day 1, day 2 and day 3-4) did not differ significantly between the hospital nonsurvivors and survivors (P = 0.08–0.45).

The discriminative power of plasma HO-1 regarding ICU and hospital mortalities was determined by ROC analysis. The AUC for the day 1 HO-1 concentration regarding ICU mortality was 0.83 (95% CI 0.73–0.93) (Fig. 12). In comparison, the AUC for the APACHE II regarding ICU mortality was 0.81 (95% CI 0.68–0.93), for the SAPS II 0.85 (95% CI 0.76–0.93), and for the day 1 SOFA 0.86 (95% CI 0.71–1.0) (Fig. 12). The best cutoff value for the day 1 HO-1 regarding ICU mortality was 7.4 ng/ml, with sensitivity of 1.00 (95% CI 0.74–1.00) and specificity of 0.58 (95% CI 0.51–0.65), positive likelihood ratio of 2.4, and correct classification rate of 0.60. The AUC for the maximum HO-1 concentration regarding hospital mortality was 0.61 (95% CI 0.52–0.71), while the first-day HO-1 value produced an AUC of 0.59 (95% CI 0.47–0.71) (Fig. 12). The AUC for the APACHE II score regarding hospital mortality was 0.76 (95% CI 0.66–0.85), for the SAPS II score 0.79 (95% CI 0.70–0.88), and for the day 1 SOFA 0.74 (95% CI 0.64–0.84) (Fig. 12).

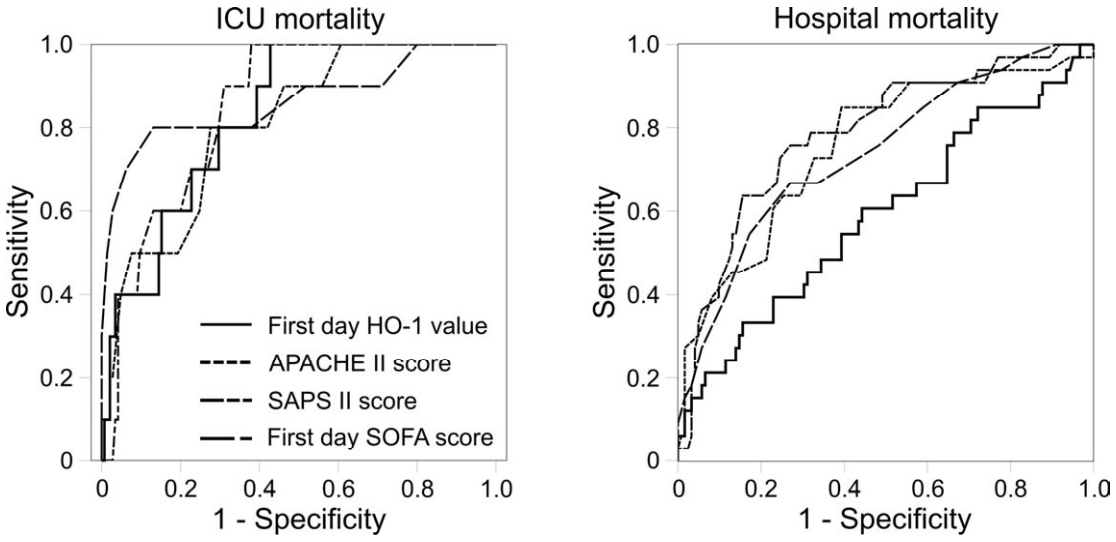

Figure 12. ROC curves for the day 1 HO-1 concentration, APACHE II score, SAPS II score and day 1 SOFA score in predicting ICU mortality and hospital mortality. (Unpublished data.) Abbreviations: APACHE II = acute physiology and chronic health evaluation II score, HO-1 = heme oxygenase-1, ROC = receiver operating characteristic, SAPS II = simplified acute physiology score II, SOFA = sequential organ failure assessment score.

Multiple regression analysis was used to investigate the factors associating independently with both ICU and hospital mortalities. All variables associated significantly with mortality were included: day 1 HO-1 concentrations, SAPS II score and SOFA score regarding ICU mortality and the maximum HO-1 concentration, SAPS II score without age, age, day 1 SOFA score, and gender regarding hospital mortality. The SAPS II score without age, age, male gender, and maximum HO-1 concentration were independently associated with hospital mortality (P = 0.001, P = 0.004, P = 0.04, P = 0.01, respectively). Only the day 1 SOFA score was independently associated with ICU mortality (P < 0.001).

6 DISCUSSION

This study shows the manifold, and for the most part salutary, effects of the HO-1/CO pathway in the heart during healing of MI and during reperfusion of the ischemic myocardium in the animal models used. In addition, this study sheds light on the role of HO-1 in critically ill patients, as well as in the subpopulation of patients with severe cardiac disease.

6.1 HO-1 expression in infarcted rat hearts (I–III)

The study I was carried out to obtain detailed data on HO-1 expression in infarcted hearts. At the time this study was conducted, the increased HO-1 expression had been shown in I/R rat and porcine hearts and in hypoxic cardiomyocytes (Maulik et al. 1996, Borger and Essig 1998, Sharma et al. 1999, Hangaishi et al. 2000, Foresti et al. 2001), but few data were available on the long-term expression of HO-1 in failing hearts (Raju et al. 1999). This study was the first to demonstrate the spatiotemporal expression of HO-1 in post-MI rat hearts (I). Similar results were obtained recently from following studies (II, III), with some additional quantitative data on HO-1 protein levels.

The results of studies I–III showed that the expression of HO-1 was induced in the infarcted hearts, especially in the infarct area and infarct border area. HO-1 expression peaked in the infarct border area at day 1 and was localized in the cardiomyocytes. HO-1 is known as a highly inducible stress-responsive protein (Otterbein and Choi 2000). Therefore, this early increase of HO-1 in the cardiomyocytes bordering the infarct area likely represents a defense response against both ischemia and ROS produced in the ischemic myocardium, and potentially promotes the survival of these cardiomyocytes. Increased HO-1 protein was detected in the cardiomyocytes of the infarct border area until week 1. At week 4, HO-1 protein was no longer detected in the cardiomyocytes, although HO-1 protein levels were still elevated in the infarct border area, according to the HO-1 ELISA results. However, the lower sensitivity of immunofluorescent staining likely explains this difference.

HO-1 is a microsomal enzyme, as originally described by Tenhunen et al. (1968, 1969) and primarily localized in the smooth endoplasmic reticulum (Maines 1988), which is the main component of the SR in cardiomyocytes. In addition to microsomes, localization of HO-1 was also reported in mitochondria (Converso et al. 2006a) and in nucleus (Lin et al. 2007a). In the present study, HO-1 showed a strong transverse and a weaker longitudinal striated pattern of staining in rat cardiomyocytes compatible with the localization of HO-1 mainly in transverse junctional SR and to a lesser extent in longitudinal SR. Interestingly, increased HO-1 protein was also detected in the intercalated discs of cardiomyocytes bordering the infarct area. The function of intercalated discs is to connect cells to each other and conduct (electrical) signals between cells through gap junctions. The localization of HO-1 in the intercalated discs suggests a possible role for HO-1 in signal conduction and it prompted us to investigate the effect of HO-1 induction on expression and distribution of cardiac gap junction protein Cx43 in study IV.

In the infarct area HO-1 expression peaked at day 3 and was highly expressed in the infiltrating monocytes/macrophages (Lakkisto et al. unpublished result) and to a lesser extent in fibroblast-like cells, whereas at week 1 HO-1 was mainly expressed in fibroblast/myofibroblasts. Likewise, Hangaishi et al. (2000) showed high levels of HO-1 in monocytes/macrophages and in myofibroblasts in response to I/R in rat hearts. The exact role of HO-1 in the infarct-invading monocytes/macrophages is not known. The preferential expression of HO-1 in anti-inflammatory human M2 macrophages was demonstrated recently (Sierra-Filardi et al. 2010).

HO-1 also contributes to the activation of human macrophages toward the anti-inflammatory M2 phenotype (Weis et al. 2009). Furthermore, HO-1-overexpressing macrophages possessed an anti-inflammatory phenotype *in vitro* and improved renal function in mouse renal I/R injury *in vivo* (Ferenbach et al. 2010). Whether HO-1 is also associated with the anti-inflammatory macrophages in the infarcted heart, and the specific role of HO-1-expressing macrophages in the healing of MI, remains to be determined in further studies. Likewise, the function of HO-1 in the fibroblasts/myofibroblasts of the infarct area is still unclear, although it is known that HO-1 inhibits apoptosis of fibroblasts and decreases proliferation of cardiac fibroblasts *in vitro* (Ferris et al. 1999, Petrache et al. 2000, Liu et al. 2006).

Study I also revealed a constant expression of HO-1 in the coronary artery walls of the rat heart and a transient expression of HO-1 in the small arterioles of the infarct border area. These results pointed to a possible role for HO-1 in maintaining perfusion and vascular homeostasis in the surrounding areas of infarction. The effects of HO-1 that may potentially contribute to the preservation of perfusion include vasodilatation (Morita et al. 1995), inhibition of endothelial cell apoptosis (Brouard et al. 2000), and inhibition of platelet aggregation by the HO reaction product CO (Brune and Ullrich 1987). In the light of current knowledge of the angiogenic effect of HO-1 (Dulak et al. 2008), and since a significant (although small) increase in capillary density was observed in the control MI hearts from week 1 to week 4 in study II, it would be tempting to speculate that the increased expression of HO-1 in the infarct border area vasculature could also partly indicate the activation of angiogenesis in the infarcted hearts.

6.2 Protection against postischemic dysfunction and ventricular fibrillation by HO-1 in ischemic/reperfused rat hearts (IV)

The cytoprotective properties of HO-1 have been studied extensively in different I/R injury models and different organs (Katori et al. 2002a). The purpose of study IV was to investigate the effect of HO-1 induction on postischemic cardiac function and I/R-induced arrhythmias. The results showed that HO-1 induction by hemin improved postischemic cardiac function and reduced VF in I/R rat hearts. Similar protection against postischemic cardiac dysfunction by HO-1 has been reported in several experimental studies. Pharmacological HO-1 induction by hemin (Clark et al. 2000b) or N-tert-butyl-α-phenylnitrone improved myocardial function after I/R in isolated perfused rat hearts (Bak et al. 2002), and cardiac-specific overexpression of HO-1 improved postischemic cardiac function in isolated transgenic mice hearts (Yet et al. 2001, Vulapalli et al. 2002). In addition to the acute cardioprotection in I/R hearts, pre-emptive cardiac HO-1 gene transfer results in long-term improvement in cardiac function in infarcted rat hearts preserving left ventricular function up to 1 year after MI (Pachori et al. 2004, Liu et al. 2006, Liu et al. 2007). The role of HO-1 reaction products in the prevention of postischemic cardiac dysfunction was not examined in this study, but others have shown that both bilirubin and CO are involved in the recovery of postischemic cardiac function in isolated rat and mouse hearts (Clark et al. 2000b, Bak et al. 2002, Bak et al. 2003).

In study I, HO-1 immunoreactivity was detected in the intercalated discs of cardiomyocytes bordering the infarct area, suggesting a role for HO-1 in signal conduction between cardiomyocytes. Therefore, the main interest in study IV was the antiarrhythmic effect of HO-1. The antiarrhythmic effect of HO-1 was also studied by Tosaki's group and they have demonstrated, using isolated perfused rat and mouse hearts, that the antiarrhythmic effect of HO-1 is mediated by CO (Bak et al. 2002, Bak et al. 2003), and exposure of isolated hearts to CO via perfusion buffer dose-dependently reduces the incidence of I/R-induced VF (Bak et al. 2005, Varadi et al. 2007). CO is known to increase cGMP levels by activating sGC (Kharitonov et al. 1995), and cGMP modulates gap junctional coupling in hamster cardiomyocytes (De Mello

1998). In addition, reduced cGMP levels are associated with increased susceptibility to I/R-induced VF in rat hearts (Pabla et al. 1995). Bak et al. (2005) reported increased cardiac cGMP levels in response to administration of CO via perfusion buffer and suggested that the antiarrhythmic effect of CO is related to increased cGMP in isolated rat hearts. In contrast, the results of study IV showed decreased cardiac cGMP levels in all I/R rat hearts regardless of pretreatment. This indicates that the antiarrhythmic effect of hemin preconditioning was independent of cGMP in this study. In addition, similar decrease of cGMP release in response to ischemia was shown earlier in isolated rat hearts by Agullo et al. (1999).

Important mecahisms leading to arrhythmias and cardiac dysfunction are ischemia-induced abnormalities in the cardiac levels of K^+, Ca^{2+}, and Na^+ ions. The cardiac levels of these electrolytes were not examined in this study, but two previous studies showed that HO-1 and CORM-3 regulate tissue K^+, Ca^{2+}, and Na^+ levels, thereby protecting the heart against I/R injury (Bak et al. 2003, Varadi et al. 2007). Thus, the cardioprotective effect of HO-1 may partly be due to the regulation of ion balance via CO. This is also supported by earlier studies showing that CO inhibits L-type Ca^{2+} channels in HEK293 cells (Dallas et al. 2009) and activates K_{Ca} channels in VSMCs (Wang and Wu 1997).

The novel finding of study IV was the modulation of expression, phosphorylation, and distribution of the predominant ventricular gap junction protein Cx43 by HO-1 induction. Gap junctions are transmembrane channels mediating intercellular communication, including electrical coupling, between neighboring cardiomyocytes. Abnormalities in gap junctions such as decreased connexin expression and alterations in gap junction distribution and phosphorylation increase the incidence of arrhythmias in both human cardiac diseases and animal models (Severs et al. 2004, 2008). The results of study IV showed in isolated rat hearts that under normoxic conditions Cx43 was phosphorylated and localized in the intercalated discs of cardiomyocytes. I/R decreased the levels and caused dephosphorylation of Cx43, as demonstrated earlier by Schulz et al. (2003) in swine hearts. In contrast, HO-1 induction prevented the I/R-related decrease in total Cx43 level, suggesting that preservation of Cx43 total level may at least partly contribute to the cardioprotective and antiarrhythmic effects of HO-1. The importance of normal Cx43 levels was demonstrated by Lerner et al. (2000) and Schwanke et al. (2002) showing increased incidence of I/R-induced ventricular arrhythmias and loss of preconditioning in heterozygous Cx43 knockout mice, due to reduced expression of Cx43.

The study IV also showed that the preservation of Cx43 total level in the I/R rat hearts by HO-1 was for the most part due to increased levels of nonphosphorylated Cx43 at serine 368 (S368). In comparison to other I/R groups, HO-1 induction resulted in greater accumulation of S368 nonphosphorylated Cx43 in both the intercalated discs and lateral plasma membranes of cardiomyocytes. Ischemia is known to cause dephosphorylation and lateral redistribution of Cx43, which are related to electrical uncoupling of cardiomyocytes and increased susceptibility to arrhythmias (Matsushita et al. 1999, Lampe et al. 2006, Solan and Lampe 2009). Since HO-1 induction decreased the incidence of VF while increasing S368 nonphosphorylated Cx43, the cardioprotective and antiarrhythmic effects of HO-1 induction may at least partly be independent of gap junctional coupling. Indeed, Li et al. (2004) showed using mouse cardiomyocytes that although Cx43 is essential in ischemic preconditioning, protection against I/R injury does not involve intercellular communication through gap junctions. One possible mechanism for the cardioprotection associated with increased nonphosphorylated Cx43 could be the inhibition of gap junction-mediated spread of cell injury due to uncoupling of cardiomyocytes (Garcia-Dorado et al. 2004). It was shown in swine and rabbit hearts that inhibition of gap junctional communication with different blockers decreases infarct size after I/R (Garcia-Dorado et al. 1997, Miura et al. 2004). Other possible mechanisms of cardioprotection related to Cx43 may also exist. Cx43 localized in cardiomyocyte mitochondria has been implicated in ischemic preconditioning in swine hearts (Boengler et al. 2005).

Furthermore, nonjunctional Cx43 hemichannels play a role in cellular volume regulation (Quist et al. 2000) and in activation of cell survival pathways (Plotkin and Bellido 2001, Plotkin et al. 2002). Whether HO-1 prevents the spread of I/R injury by causing uncoupling of cardiomyocytes or modulates the Cx43-associated protective mechanisms that are independent of gap junctional coupling remains to be determined in further studies.

Multiple putative phosphorylation sites of Cx43 exist. Phosphorylation of serine residues, including S368, decreases the permeability of Cx43 hemichannels, i.e. decreases intercellular communication (Lampe et al. 2000, Bao et al. 2004, Solan and Lampe 2009). Under physiological conditions and in response to ischemic preconditioning, Cx43 is predominantly phosphorylated, keeping the gap junctions in a closed state, whereas ischemia causes dephosphorylation of Cx43 and opening of gap junctions, contributing to the spread of tissue injury (Schulz et al. 2003). Based on this, one would expect impaired cardiac function and increased incidence of VF associating with increased S368 nonphosphorylated Cx43 in study IV. Since this was not the case, we speculate that increase in S368 nonphosphorylated Cx43 by HO-1 may enhance intercellular communication and electrical coupling, thereby decreasing the incidence of VF. Vetterlein et al. (2006) showed in I/R rat hearts that ischemic preconditioning caused redistribution of Cx43 in the lateral plasma membrane of cardiomyocytes, associating with improved survival of cardiomyocytes during subsequent prolonged ischemia. They did not specify the phosphorylation status of Cx43, but Hund et al. (2007) showed that ischemia increases phosphorylation of Cx43 at S368, whereas ischemic preconditioning prevents phosphorylation in isolated perfused mouse hearts. This suggests indirectly that S368 nonphosphorylated Cx43 could be involved in the beneficial effects of ischemic preconditioning, and also of hemin preconditioning. However, the significance of S368 nonphosphorylated Cx43 in the hemin-preconditioned hearts remains unclear, since contrasting results of decreased phosphorylation of Cx43 at S368 have also been reported in ischemic rat hearts (Axelsen et al. 2006, Matsushita et al. 2006). Therefore, the specific role of S368 nonphosphorylated Cx43 in cardioprotection by HO-1 remains to be evaluated in further studies.

Taken together, the data on expression, distribution and phosphorylation of Cx43 suggest that HO-1 may decrease the incidence of arrhythmias and improve postischemic cardiac function partially by preserving cardiac total Cx43 levels in I/R hearts. HO-1 may also inhibit the spread of cell injury in I/R hearts by increasing S368 nonphosphorylated Cx43 that causes uncoupling and lateralization of Cx43. However, the cardioprotective and antiarrhythmic effects of HO-1 may also be independent of gap junctional coupling.

In humans, the role of HO-1 in cardiac arrhythmias is unclear. Increased atrial HO-1 mRNA and protein levels have been shown in patients with chronic atrial fibrillation (Bukowska et al. 2008, Corradi et al. 2008). The induction of HO-1 in these patients may result from increased oxidative stress in the atrial myocardium (Bukowska et al. 2008, Corradi et al. 2008). It is also well known that CO poisoning causes cardiac arrhythmias, which are a major cause of death in patients with CO poisoning (Dolan 1985). In contrast, the effect of low doses of CO on cardiac arrhythmias is controversial. Urban air pollution containing CO is associated with cardiac arrhythmias (Link and Dockery 2010), whereas CO exposure of coronary artery patients causing 3–6% COHb levels does not increase the frequency of ventricular arrhythmias (Hinderliter et al. 1989, Dahms et al. 1993). Since HO-1 and low doses of CO efficiently inhibit cardiac arrhythmias in rodents, they may also have therapeutic potential in humans. HO-1 gene therapy and CORMs will likely be the most useful therapeutic strategies to investigate in humans, presuming that they turn out to be safe for patients.

It should be noted that study IV has some limitations. The most important limitation is that the cardiac mRNA, protein, and cGMP levels were measured and Cx43 immunohistochemical analysis performed at the end of the 120-min reperfusion. Therefore, the biochemical and

immunohistochemical results represent the conditions at that time point, but the effect of HO-1 induction on cGMP and Cx43 levels and Cx43 localization during ischemia or early reperfusion cannot be evaluated, based on this study. Since the cGMP levels were similar in all I/R groups at the 120-min reperfusion, it is likely that hemin preconditioning had no significant effect on cardiac cGMP content before or during the ischemia. Instead, phosphorylation of Cx43 may change rapidly (Crow et al. 1990, Boengler et al. 2006). Therefore, both phosphorylation status and localization of Cx43 during ischemia and early reperfusion may have been different from those at 120 min of reperfusion. In addition, the infarct size was not measured in this study, although HO-1 induction may have protected the heart partly by decreasing infarct size.

6.3 The myriad effects of HO-1 and CO on the recovery from rat myocardial infarction (II, III)

The results of studies II and III showed that HO-1 and CO promoted the healing of infarcted rat hearts by multiple mechanisms. The study II was the first to show that CO activated c-kit+ stem/progenitor cells and promoted vasculogenesis and myocardial regeneration by regulating the expression of the angiogenic factors HIF-1α, SDF-1α, and VEGF-B. It also revealed differential mechanisms of action for HO-1 and CO in cardiac regeneration, since HO-1 appeared to promote angiogenesis. The study III demonstrated for the first time the effects of HO-1 induction on proliferation and repair of cardiomyocytes in the infarcted hearts and, furthermore, the effects of HO-1 on proliferation of cardiac fibroblasts and cardiac fibrosis *in vivo*. In addition, the role of CO in cellular and extracellular remodeling is clarified here.

6.3.1 Neovascularization and myocardial regeneration (II)

An intriguing mechanism for the healing of MI has emerged during the last decade, since it has become evident that the heart is a regenerating organ. The adult mammalian heart contains a population of resident c-kit+ CSCs with the potential for regenerating infarcted myocardium when activated with growth factors or delivered into the infarcted hearts (Beltrami et al. 2003, Dawn et al. 2005, Linke et al. 2005, Tillmanns et al. 2008). In addition, paracrine factors secreted from stem/progenitor cells and from the ischemic myocardium contribute significantly to cardiac regeneration (Gnecchi et al. 2008, Smart and Riley 2008). Estimates of the cardiomyocyte renewal rate in humans vary from 0.45–1% per year to 22% per year (Bergmann et al. 2009, Kajstura et al. 2010).

Although the cytoprotective and proangiogenic roles of HO-1 are well established (Dulak et al. 2008), few studies have investigated the role of HO-1 in cardiac cell therapy or cardiac regeneration. HO-1 transfection improves survival of transplanted MSCs in ischemic mouse hearts (Tang et al. 2005, Zeng et al. 2008a). HO-1-overexpressing MSCs protected against cardiomyocyte apoptosis and cardiac dysfunction, increased capillary density, and decreased infarct size in infarcted rat hearts (Zeng et al. 2008a, Tsubokawa et al. 2010). Similar effects were shown in infarcted rat hearts injected with cell culture supernatant of HO-1-transfected MSCs, suggesting that the paracrine factors secreted by HO-1-modified MSCs mediate the beneficial effects of these cells in the heart (Zeng et al. 2008b). In addition to our results (II), there is only one other study by Lin et al. (2008) investigating the effect of HO-1 on cardiac regeneration. They showed that HO-1 gene transfer promotes neovascularization in ischemic mouse hearts by markedly increasing recruitment of circulating c-kit+ and CD34+ stem/progenitor cells and increasing the levels of VEGF and SDF-1 (Lin et al. 2008). We showed that pharmacological HO-1 induction caused only small increases of c-kit+ cells in the infarcted rat hearts despite increased SDF-1α levels, suggesting that HO-1 rather induced angiogenesis in

this study (II). The difference between these results may be due to the different species used, or to different methods for increasing HO-1 expression.

The interesting novel finding in study II was the strong activation of c-kit+ stem/progenitor cells and formation of new arteries in the infarcted rat hearts by CO. CO increases circulating endothelial progenitor cells and enhances endothelial repair after vascular injury in mice and rabbits (Lin et al. 2009, Wu et al. 2009, Wegiel et al. 2010). The CO-induced c-kit+ cells were negative for the hematopoietic stem cell marker CD34 (II), and in later studies negative for Sca-1 which is another marker of bone marrow-derived progenitor cells (Lakkisto et al., unpublished result). These results indicate that the CO-activated c-kit+ cells were more likely of local origin, not derived from bone marrow. Resident CSCs have the potential to differentiate into all cardiac cell types: cardiomyocytes, endothelial cells, and VSMCs and form new capillaries and arteries (Beltrami et al. 2003, Linke et al. 2005, Tillmanns et al. 2008). In study II, CO promoted a substantial differentiation of c-kit+ cells into VSMCs and to a lesser extent into cardiomyocytes, whereas few endothelial cell-differentiating cells were found. In concert, CO increased vascular density, especially the number of intermediate and large arteries, but failed to increase capillary density. Furthermore, increased proliferation of cardiomyocytes was observed in the infarct border area coincident with the increase in c-kit+ cells, potentially representing the proliferation of newly formed cardiomyocytes derived from c-kit+ cells. These results suggest that CO may also activate EDPCs. EDPCs are crucial to the development of coronary vessels and the cardiac fibrous skeleton during embryogenesis (Winter and Gittenberger-de Groot 2007) and differentiate into SMCs, but not into endothelial cells *in vitro* (Wada et al. 2003, van Tuyn et al. 2007). In support of the EDPC hypothesis, adult c-kit+ human and mouse EDPCs also give rise to cardiomyocytes (Limana et al. 2007) and Tbx18+ mouse EDPCs to cardiomyocytes and coronary SMCs, but not endothelial cells (Cai et al. 2008). In further studies, some c-kit+ cells were positive for Tbx18 suggesting that the c-kit+ cells in study II may have originated from the epicardium (Lakkisto et al. unpublished result).

The inability of CO to increase capillary density in the infarcted hearts is in contrast with previous *in vitro* studies showing that CO mediates the proangiogenic properties of HO-1 (Jozkowicz et al. 2003, Li Volti et al. 2005). This may be due to different conditions in the heart *in vivo* compared with *in vitro* endothelial cell cultures. CO may also have a function in the heart that is different from that of the vasculature, since CO accelerates endothelial cell proliferation *in vitro* and endothelial repair after carotid artery injury *in vivo* (Lin et al. 2009, Wegiel et al. 2010). It is also noteworthy that the effects of CO or HO-1 on proliferation of endothelial cells in hypoxic/ischemic conditions are unknown. Thus CO may function differently in ischemic conditions compared with physiological conditions.

6.3.2 Expression of angiogenic factors

No c-kit+ cells were found in sham-operated rat hearts, suggesting that the hypoxia-induced factors secreted from the infarct area were involved in the activation of c-kit+ cells by CO. The results of study II showed that CO increased HIF-1α, SDF-1α, and VEGF-B levels in the infarct area, whereas VEGF-A levels did not differ significantly between groups. HIF-1 is a transcription factor that initiates the cellular response to hypoxia (Weidemann and Johnson 2008). HIF-1 directly induces the expression of several angiogenic factors, including VEGF and SDF-1, and induces angiogenesis (Forsythe et al. 1996, Kelly et al. 2003, Ceradini et al. 2004, Weidemann and Johnson 2008). CO increases HIF-1α levels in macrophages *in vitro* and in transplanted rat kidneys *in vivo,* and protects against I/R injury in lungs and kidneys via an HIF-1-dependent mechanism (Chin et al. 2007, Faleo et al. 2008). CO may activate HIF-1α by causing more severe ischemia in the infarcted myocardium. However, Chin et al. (2007) showed in macrophages and in lung I/R injury that CO activates and stabilizes HIF-1α by causing a

transient increase in ROS arising from mitochondria. Interestingly, HO-1 may contribute to the HIF-1-mediated response to CO, since HIF-1 is known to regulate the expression of HO-1 (Ockaili et al. 2005). Furthermore, the cardioprotective effects of the HIF-1α subunit were dependent on HO-1 activity in mouse hearts (Czibik et al. 2009).

Study II is the first to show that CO increases the levels of VEGF-B in infarcted rat hearts. VEGF-B is a member of the VEGF family that promotes angiogenesis and arteriogenesis selectively in ischemic hearts (Li et al. 2008, Lähteenvuo et al. 2009). VEGF-B is also implicated in formation of coronary arteries during embryogenesis (Tomanek et al. 2002, 2006) and it promotes survival of cells, including cardiomyocytes and blood vessels, and regulates energy metabolism by regulating fatty acid uptake in different animal models (Zhang et al. 2009, Hagberg et al. 2010, Pepe et al. 2010). However, the significance of increased VEGF-B in CO-induced vasculogenesis remains to be determined in further studies.

VEGF-A was not significantly involved in either HO-1- or CO-induced neovascularization in this study. The majority of previous studies have demonstrated increased VEGF-A levels in response to HO-1 induction, but contrasting results have also been reported. HO-1 induction increases VEGF-A production in endothelial cells and VSMCs by a CO-dependent mechanism (Dulak et al. 2002, Jozkowicz et al. 2003). HO-1 transfection increases VEGF-A levels in MSCs (Tsubokawa et al. 2010). In addition, cardiac HO-1 gene transfer increased VEGF-A levels in ischemic mouse hearts and systemic HO-1 gene delivery in mouse serum (Lin et al. 2008, 2009). Several factors also increase VEGF-A via an HO-1-dependent mechanism (Kim et al. 2006, Samuel et al. 2010, Shibuya et al. 2010). In contrast, Lin et al. (2009) showed that CO does not increase VEGF-A levels in mouse serum. In addition, short exposure of keratinocytes to the HO-1 inducer heme increases VEGF-A expression, while longer exposure may decrease VEGF-A expression (Jazwa et al. 2006). Iron released in the HO reaction may also inhibit VEGF-A synthesis (Dulak et al. 2002). The lack of significant VEGF-A response in study II may have been due to a different HO-1 inducer and different methods for CO delivery compared with previous studies or to the different time points studied.

In study II, both HO-1 induction and CO pretreatment caused a sustained increase in SDF-1α in the infarcted myocardium. SDF-1α is a chemokine playing an important role in the mobilization and homing of stem/progenitor cells into the infarcted heart (Abbott et al. 2004, Elmadbouh et al. 2007, Zhao et al. 2009), in the survival of hypoxic/reoxygenated cells (Hu et al. 2007), and in neovascularization and myogenesis of the infarcted hearts (Elmadbouh et al. 2007, Zhao et al. 2009). SDF-1α levels increase naturally in response to myocardial ischemia, but decrease by 7 days post-MI in rats (Askari et al. 2003). The decline in SDF-1α appears to occur at a critical time, because Fransioli et al. (2008) showed that c-kit+ stem cells naturally appear in the infarct area 1–2 weeks after MI in mice. The decline in SDF-1α by week 1 may thus partly explain the inefficient cardiac repair after MI. Indeed, sustained elevation of SDF-1α by delivery of SDF-1α–overexpressing fibroblasts or by cardiac SDF-1α gene transfer increases stem cell accumulation in the ischemic heart and improves cardiac function in mouse and rat hearts (Askari et al. 2003, Abbott et al. 2004). Deshane et al. (2007) showed that SDF-1 promotes angiogenesis in endothelial cells and aortic rings *in vitro* and in Matrigel plugs *in vivo* via an HO-1-dependent mechanism. In contrast, Lin et al. (2008, 2009) showed that cardiac HO-1 gene transfer promoted neovascularization in infarcted mouse hearts via SDF-1- and VEGF-dependent mechanisms, and systemic HO-1 gene delivery and low-dose CO exposure increased SDF-1 levels in mouse serum. The data shown by Lin et al. (2008, 2009) are consistent with the sustained increase of SDF-1α by HO-1 induction and CO pretreatment in study II. The up-regulation of SDF-1α by HO-1 and CO may provide new therapeutic approaches to enhance repair of infarcted hearts.

6.3.3 Proliferation and survival of cardiomyocytes

The proliferation of cardiomyocytes is one piece of a puzzle in myocardial regeneration. The heart contains a population of small proliferative cardiomyocytes, and the proliferation of cardiomyocytes is increased in response to MI in both human hearts and experimental animal models (Beltrami et al. 2001, Yuasa et al. 2004, Chen et al. 2007). However, this increase as such is not sufficient for repair of infarcted hearts. Enhancement of cardiomyocyte proliferation by cell cycle regulators or growth factors is needed to decrease infarct size and to improve myocardial function after MI (Engel et al. 2006, Woo et al. 2006).

HO-1 regulates the cell cycle in a cell type-specific manner and the effect of HO-1 on cell proliferation is mediated by CO (Peyton et al. 2002, Song et al. 2002, Wegiel et al. 2008, Wegiel et al. 2010). Previous *in vitro* studies have shown that HO-1 increases proliferation of endothelial cells, whereas it decreases proliferation of several other cell types: vascular and airway SMCs, cardiac and lung fibroblasts, and pancreatic stellate cells (Li Volti et al. 2002, Peyton et al. 2002, Song et al. 2002, Zhou et al. 2005, Liu et al. 2006, Schwer et al. 2010). Due to the role of HO-1 in regulation of the cell cycle in several other cell types, we investigated whether HO-1 promotes healing of infarcted hearts by regulating the proliferation of cardiomyocytes. The study III is the first to show the temporal and spatial effects of HO-1 induction on proliferation of cardiomyocytes in postinfarction rat hearts. The role of CO in the proliferation of cardiomyocytes is also shown here. Taken together, the results suggest that HO-1 and CO may potentially regulate the cardiomyocyte cell cycle in infarcted hearts. However, the overall effect of HO-1 and CO on proliferation of cardiomyocytes appears to be dependent on the milieu of cardiomyocytes at a given time and location in the postischemic heart.

The results showed that HO-1 induction increased the number of Ki67+ proliferating cardiomyocytes in the infarct border area during the first few days after MI, whereas this effect was not seen in the CO donor-pretreated group. This suggests that HO-1 may increase proliferation of cardiomyocytes in the infarct border area by protecting the cardiomyocytes and maintaining cellular homeostasis in the ischemic myocardium via the antioxidative activity of another HO reaction product, bilirubin. Bilirubin decreases infarct size and improves cardiac function after I/R in rats (Clark et al. 2000b). In contrast to HO-1 induction, CO increased the number of proliferating cardiomyocytes in the infarct border area concomitantly with the increase in c-kit+ stem/progenitor cells at week 4. However, this effect was only seen in CO-pretreated hearts, suggesting that it represented proliferation of newly formed cardiomyocytes derived from c-kit+ cells rather than specific regulation of the cardiomyocyte cell cycle by CO.

In contrast to the infarct border area, HO-1 inhibited proliferation of cardiomyocytes under the supposedly normal conditions in the remote myocardium (study III) and a similar effect was seen in CO donor-pretreated hearts. This suggests indirectly that HO-1 may inhibit progression of the cardiomyocyte cell cycle under physiological conditions via CO. Previous *in vitro* studies showing that HO-1 inhibits proliferation of other muscle cell types, VSMCs, and airway SMCs support this hypothesis (Peyton et al. 2002, Song et al. 2002). However, further studies in cardiomyocytes *in vitro* are needed to confirm the inhibitory effect of the HO-1/CO pathway on cardiomyocyte proliferation under physiological conditions and evaluate the effects of HO-1 and its reaction products on cardiomyocyte proliferation under hypoxic conditions. Nevertheless, these opposing spatiotemporal effects of HO-1 and CO on cardiomyocyte proliferation are likely to result in the enhanced recovery from MI.

Limiting the expansion of the infarct by protecting the cardiomyocytes is equally important in the recovery from MI as myocardial regeneration. The results of study III demonstrated that HO-1 improves survival of cardiomyocytes, as indicated by increased repair of cardiomyocyte

DNA and decreased apoptosis of cardiomyocytes. The cardioprotection is partially mediated by CO.

HO-1 increased the repair of cardiomyocyte DNA in the infarct border area during the first few days after MI, as indicated by the increased number of PCNA+ cardiomyocytes. CO did not affect the number of PCNA+ cardiomyocytes during these first days after MI, pointing again to the role of bilirubin in maintaining cellular homeostasis in the early phase of infarct healing. In contrast, CO increased the number of PCNA+ cardiomyocytes in the infarct border at week 4. Whether this increase in cardiomyocyte repair is associated with CO-induced cardiac regeneration or pathological changes in the CO-pretreated hearts remains to be determined.

In chronic heart diseases, the increase in PCNA+ cardiomyocytes may also serve as a marker of stress and the development of heart failure. Increased repair of cardiomyocyte DNA was demonstrated in patients with dilated cardiomyopathy and severe heart failure while implantation of a left ventricular assist device reduced PCNA expression (Francis et al. 1999, Bartunek et al. 2002). In infarcted rat hearts, both HO-1 induction and CO pretreatment attenuated the increase in PCNA+ cardiomyocytes at week 4 in the remote myocardium, suggesting that HO-1 may protect against adverse ventricular remodeling and development of heart failure in the late phase of infarct healing via CO, if increased PCNA expression is considered as a marker of heart failure.

The antiapoptotic action of HO-1 is well characterized in different cells and tissues, including cardiomyocytes. HO-1 gene transfer inhibits angiotensin II-mediated apoptosis in rat cardiomyocytes *in vitro* (Foo et al. 2006). Cardioselective overexpression of HO-1 inhibited cardiomyocyte apoptosis in I/R mouse hearts and in the failing mouse hearts (Vulapalli et al. 2002, Wang et al. 2010). In addition, HO-1 induction by CoPPIX protects transplanted rat hearts from I/R injury and decreases cardiomyocyte apoptosis (Katori et al. 2002b). Consistent with previous studies, HO-1 decreased cardiomyocyte apoptosis in study III. A similar reduction of cardiomyocyte apoptosis was found in the CO donor-pretreated hearts, indicating that the antiapoptotic effect of HO-1 is mediated by CO, as shown previously in several studies (Sato et al. 2001, Uemura et al. 2005, Piantadosi et al. 2008, Wang et al. 2010).

6.3.4 Cardiac fibrosis and extracellular matrix components (III)

Cardiac fibroblasts are the most numerous cell type in the rat heart, contributing to cardiac structure and function (Camelliti et al. 2005, Banerjee et al. 2007, Porter and Turner 2009). Fibroblasts are equally important cells in the healing of infarcted heart as cardiomyocytes, due to their key role in post-MI ventricular remodeling (Porter and Turner 2009). Fibroblasts are activated in response to acute MI: they migrate into the infarct area, proliferate, and participate in the repair of injury by forming a fibrous scar (Sun and Weber 2000, Porter and Turner 2009). However, improper activation of fibroblasts and increased fibrosis in the noninfarcted myocardium together with apoptosis and hypertrophy of cardiomyocytes lead to the development of heart failure (Sun and Weber 2000, Porter and Turner 2009).

The results of study III suggested that HO-1 induction may attenuate the fibrotic response to MI, since a lower total number of cells and lower numbers of vimentin+ fibroblastlike cells were found in the infarct area, along with decreased proliferations of cells in the infarct area and in the infarct border area. Attenuated proliferation of fibroblasts was also suggested by lower levels of vimentin protein (an indirect measure of fibroblast number) at week 4. In contrast to HO-1 induction, the unpublished results of CO-pretreated hearts showed that CO did not affect the number or proliferation of all cells or fibroblasts alone. Consistent with these results, Liu et al.

(2006) showed earlier that HO-1 gene transfer inhibits proliferation of rat cardiac fibroblasts *in vitro* and decreases the infiltration of myofibroblasts in the infarct area *in vivo*.

Taken together the results of cardiac fibrosis and expression of the extracellular matrix components HO-1 and CO appear to modulate the biochemical function of fibroblasts by divergent mechanisms. HO-1 induction decreased the levels of Coll1a1 mRNA and procollagen type I protein at week 1, and the extent of perivascular fibrosis at week 4. HO-1 inhibits fibrosis in kidneys, liver, and lungs (Tsuburai et al. 2002, Gaedeke et al. 2005, Tsui et al. 2005). However, HO-1 had no effect on the extent of interstitial fibrosis or on the expression of Coll3a1 or fibronectin mRNAs that are usually increased in interstitial fibrosis. In addition, no differences were found in cardiomyocyte cross-sectional area (III). In contrast, HO-1 induction by hemin attenuated left ventricular hypertrophy and fibrosis in adult spontaneously hypertensive rats, and HO-1 gene transfer decreased interstitial collagen at 1.5 and 3 months after I/R injury in rat hearts (Liu et al. 2007, Ndisang and Jadhav 2009). These contrasting results are likely due to the different models and time points studied. It is likely that week 4 post-MI is too early to detect differences in interstitial fibrosis or in cardiomyocyte hypertrophy. In addition, increased interstitial fibrosis is more characteristic of hypertension.

In contrast to HO-1 induction, CO had no significant decreasing effect on the extent of perivascular or interstitial fibrosis or on the levels of procollagen I and vimentin proteins. Instead, CO activated the gene expression of fibronectin, Coll1a1, and Coll3a1 at week 4. These results suggest that CO does not mediate the antifibrotic effect of HO-1 in the heart. This is in contrast with previous studies showing that CO suppresses bleomycin-induced fibrosis in lungs and inhibits development of renal fibrosis in obstructive nephropathy (Zhou et al. 2005, Wang et al. 2008). However, the CO may have varying effects on different organs, since Andre et al. (2010) showed that chronic CO exposure by CO-enriched air increased interstitial and perivasular fibrosis in healthy rat hearts. It would also be tempting to speculate that the increased gene expression of major extracellular matrix proteins at week 4 could be associated with CO-activated stem/progenitor cells. As discussed above, the CO-activated c-kit+ cells are likely of epicardial origin. EDPCs play a crucial role in formation of the fibrous skeleton of the heart (Winter and Gittenberger-de Groot 2007), and it is currently believed that the majority of cardiac fibroblasts originate from EDPCs (Snider et al. 2009). Further studies are needed to address the effect of CO on cardiac fibroblasts and fibrosis, because increased cardiac fibrosis as a side effect would clearly lower the possible therapeutic value of CO.

The expression of TGF-β and CTGF was measured to evaluate whether the effects of HO-1 and CO on cardiac fibrosis are mediated by regulating the expression of these factors. TGF-β promotes cardiomyocyte hypertrophy and increases fibrosis in the late phases of infarct healing (Bujak and Frangogiannis 2007). However, it plays an important anti-inflammatory role in the early phase of infarct healing (Bujak and Frangogiannis 2007). TGF-β also contributes to limiting the expansion of inflammation into the noninfarcted myocardium (Frangogiannis et al. 2005). CTGF is one of the downstream mediators of the profibrotic effect of TGF-β.

The results of study III showed that HO-1 induction increased the gene expression of TGF-β1 in the infarct area and CTGF in the remote area at day 3. In addition, CO-pretreated hearts showed similar increases in TGF-β1 expression at day 3. These results point to the anti-inflammatory roles of HO-1 and CO and suggest that in the early phase of infarct healing the beneficial effects of HO-1 may be mediated in part by CO and the anti-inflammatory cytokine TGF-β1. Furthermore, the simultaneous transient increase in CTGF expression may be associated with the progression of infarct healing from the inflammatory phase to the proliferative phase characterized by suppression of the inflammatory response and formation of the fibrous scar. TGF-β and IL10 mediate the anti-inflammatory action of HO-1 against ovalbumin-induced airway inflammation in mice (Xia et al. 2007). In addition, CO protects macrophages against

anoxia/reoxygenation-induced apoptosis by increasing TGF-β via Hif-1α (Chin et al. 2007). In contrast, HO-1 induction by heme arginate decreased cardiac TGF-β levels and fibrosis in DOCA-salt hypertension (Jadhav and Ndisang 2009). HO-1 did not affect TGF-β1 expression at the late phase of infarct healing, but it significantly decreased the expression of CTGF at week 4 in the infarct area and in the remote area (III). This suggests that in the late phases of infarct healing, HO-1 may protect against fibrosis by down-regulating CTGF. Unlike HO-1 induction, CO increased both TGF-β1 and CTGF mRNA in the infarct area at week 4. This suggests that TGF-β1 and CTGF may mediate the increased expression of fibronectin, Coll1a1, and Coll3a1 mRNAs by CO.

6.3.5 Infarct size (II, III)

Despite the several beneficial effects of both HO-1 and CO in post-MI hearts, no significant effect on infarct size was found. A trend for smaller infarcts was found in HO-1-induced hearts, suggesting that HO-1 may have prevented the expansion of ischemic injury by promoting angiogenesis in the infarct area and survival and proliferation of cardiomyocytes in the infarct border area. Recently, Wang et al. (2010) showed that the infarct size was similar in cardiac-restricted HO-1-transgenic mice compared with nontransgenic mice and also in CORM-3-pretreated mice compared with control mice, although both HO-1 overexpression and CORM-3 treatment alleviated ventricular remodeling and improved cardiac function 4 weeks after LAD ligation. Therefore, it appears that the salutary effects of HO-1 and CO do not always result in decreased infarct size in the permanent LAD ligation model. In addition, week 4 may be too early to detect improvement in infarct size, especially in CO-pretreated hearts. It is possible that the regeneration process has recently been initiated in CO pretreated hearts at week 4, because vascular density was increased and the greatest accumulation of c-kit+ cells was found at that time point. In addition, CO promoted mainly formation of new arteries, while more efficient formation of new cardiomyocytes may be needed to decrease infarct size.

6.3.6 Limitations of the myocardial infarction studies (II, III)

The MI studies have some limitations. One of the major limitations is the use of CoPPIX to induce HO-1 and methylene chloride as a CO donor. Metalloporphyrins, including CoPPIX, may have nonspecific effects unrelated to HO-1 induction or inhibition. Likewise, in comparison to new CORMs, methylene chloride may also have nonspecific effects. Another major limitation is the lack of echocardiography measurements. As discussed above, HO-1 and CO may improve cardiac function and prevent adverse ventricular remodeling without affecting infarct size. A third possible limitation is the use of HO-1 induction and CO donors as pretreatments, whereas treatments administered post-MI may be more clinically relevant.

6.3.7 Therapeutic potential of HO-1 and CO in myocardial infarction

Taken together, results of studies II and III show that HO-1 and CO have numerous beneficial effects in infarcted rat hearts. They promote neovascularization and cardiac regeneration, and modulate cellular and extracellular remodeling after MI. In humans, HO-1 expression is increased in failing hearts (Grabellus et al. 2002) and in the atrial myocardium of atrial fibrillation patients (Corradi et al. 2008), indicating that HO-1 is induced in response to stress in the human heart, similar to the situation in rat hearts. Furthermore, low-dose CO inhalation had anti-inflammatory effects and improved responsiveness to metacholine in a pilot study of patients with chronic obstructive pulmonary disease (COPD) (Bathoorn et al. 2007). Therefore,

therapeutic induction of HO-1 or administration of HO reaction products could potentially enhance recovery and repair of the post-MI human hearts as well. However, additional studies, e.g. in the swine MI model, are warranted to confirm the beneficial effects of HO-1 and CO in larger animals before clinical studies.

6.4 Role of HO-1 in critically ill patients – focus on patients with cardiac disease

HO-1 is highly induced by various stresses, especially oxidative stress, which is a common feature in the critically ill, regardless of the underlying disease. In addition, *HO-1* polymorphisms affect the transcriptional activity of the *HO-1* gene and are accociated with different clinical conditions, e.g. outcome of organ transplantation and restenosis after peripheral angioplasty and coronary stenting (Hirai et al. 2003, Exner et al. 2004, Schillinger et al. 2004, Buis et al. 2008). The study V was conducted to investigate the role of HO-1 plasma levels and *HO-1* polymorphisms in critically ill patients and evaluate whether measuring HO-1 levels would be of use in the care of these patients. In addition, the role of HO-1 in the subgroup of cardiac patients is further discussed here.

The *HO-1* GT_n repeat length polymorphism was evaluated together with the *HO-1* -413A/T and +99G/C SNPs for the first time (V). The frequencies of the *HO-1* GT_n repeat length alleles and the *HO-1* -413A/T and +99G/C alleles were consistent with the frequencies in previous studies with Caucasian patient populations (Gulesserian et al. 2005, Turpeinen et al. 2007, Lublinghoff et al. 2009). In study V, the +99C allele was in perfect LD with the long GT allele. Similar LD between the GT_n and the +99G/C polymorphism was reported earlier in one study (Gulesserian et al. 2005). The transcriptional activity of the *HO-1* gene decreases with increasing numbers of GT_n repeats (Hirai et al. 2003, Brydun et al. 2007, Song et al. 2009a). However, the functional importance of the +99G/C polymorphism remains to be determined in further studies. LD between the GT_n microsatellite polymorphism and -413A/T SNP was also reported in several studies (Buis et al. 2008, Sheu et al. 2009, Song et al. 2009a), and greater functional importance of the -413A/T polymorphism over the GT_n repeat length polymorphism has been suggested in a few studies (Ono et al. 2004, Buis et al. 2008). The results of this study suggested greater significance of the GT_n repeat length polymorphism (V). It must be noted that in previous studies, the GT_n repeat length has been divided into two or three categories, with the lowest L allele length varying from 26 to 33 repeats. This inconsistency in the classification of GT_n repeat lengths may have substantially affected interpretation of the results and of the functional importance of this polymorphism.

The results of study V showed that the -413T/GT(L)/+99C haplotype independently affected the day 1 plasma HO-1 concentrations. The HO-1 plasma levels were significantly lower in patients with the -413T/GT(L)/+99C haplotype, which is in line with previous studies showing lower transcriptional activity of the *HO-1* gene with increasing numbers of GT_n repeats (Hirai et al. 2003, Brydun et al. 2007, Song et al. 2009a). The short GT_n associated with better outcome in many diseases (Kaneda et al. 2002, Chen et al. 2004, Mustafa et al. 2008). Therefore, it was surprising that the -413T/GT(L)/+99C haplotype was associated with lower appearance of MOD in study V. However, Sheu et al. (2009) also showed that the long GT allele protected against the acute respiratory distress syndrome and Melley et al. (2007) suggested an optimal range of HO-1 induction in critically ill patients, based on COHb measurements. However, which of these is protective against MOD, the -413T/GT(L)/+99C haplotype or the lower plasma HO-1 concentration, remains to be determined in further studies.

No association was found between *HO-1* polymorphisms and ICU or hospital mortalities or disease severity scores throughout the study population or in the subgroups, including the group

of 71 cardiac patients. The lower appearance of MOD and lower HO-1 plasma levels in patients with the -413T/GT(L)/+99C haplotype may explain this, because HO-1 plasma levels were higher in the ICU and hospital nonsurvivors than in survivors, and higher HO-1 plasma levels were associated with MOD and more severe disease. It is also noteworthy that in a large number of studies, *HO-1* polymorphisms were not associated with the disease studies or the outcome (Tiroch et al. 2007, Turpeinen et al. 2007, Lublinghoff et al. 2009).

The HO-1 plasma levels were significantly higher in all critically ill ICU patients than in healthy controls (V). Patients with pancreatitis or bacterial infection had the highest HO-1 concentrations. Pancreatitis is characterized by necrosis of pancreatic tissue and activation of a systemic inflammation response (Pandol et al. 2007). Patients with cardiac disease also had relatively high HO-1 plasma levels, although not as high as patients with pancreatitis or bacterial infection. Nevertheless, HO-1 appears to be induced in cardiac diseases, such as acute MI, similar to its up-regulation in experimental MI (I–III). These results also suggest that in cardiac diseases HO-1 induction may be more local than systemic, thereby resulting in lower HO-1 plasma levels than in pancreatitis and severe sepsis, which are characterized by robust systemic inflammation. HO-1 is an intracellular protein, and increased plasma HO-1 may originate from injured tissues and cells or from inflammatory cells, especially monocytes and macrophages, or both. Increased HO-1 immunoreactivity was earlier shown in failing hearts (Grabellus et al. 2002), as has increased HO-1 mRNA and protein in the atrial myocardium of patients with chronic atrial fibrillation (Corradi et al. 2008). Eide et al. (2008) showed that pregnant women with pre-eclampsia have increased HO-1 mRNA and protein expression in decidua basalis concomitant with elevated serum HO-1 levels. Patients with silicosis also have increased HO-1 immunoreactivity in the lungs along with increased serum HO-1 levels (Sato et al. 2006). On the other hand, HO-1 expression is increased in circulating monocytes in various acute inflammatory illnesses in children (Yachie et al. 2003), and in circulating monocytes and lymphocytes of patients with coronary artery disease, which also supports the release of HO-1 from inflammatory cells (Chen et al. 2009).

Based on the results of study V, the role of increased HO-1 levels in critically ill patients remains unclear. Increased expression of HO-1 is commonly considered to protect against stress and tissue injury (Otterbein and Choi 2000). HO-1 protects the renal tubuli from oxidative injuries in various renal diseases (Morimoto et al. 2001) and rat liver against I/R injury and improves microcirculation (Schmidt et al. 2007). HO-1 also has cytoprotective effects on the heart and vasculature (Idriss et al. 2008) and on the lungs (Jin and Choi 2005). However, the lower frequency of MOD in patients with the -413T/GT(L)/+99C haplotype and lower plasma HO-1 levels suggests that increased HO-1 may also have detrimental effects. Melley et al. (2007) showed that both low minimum and high maximum blood COHb levels were associated with increased ICU mortality. These results suggest that there may be a therapeutic window for HO-1 expression. High levels of HO reaction products may indeed have adverse effects. The release of pro-oxidative free iron may cause adverse effects as well as increased bilirubin, and high level of CO impairs O_2 transport and tissue O_2 delivery (Horvath et al. 1975, Emerit et al. 2001, Smithline et al. 2003, Immenschuh et al. 2007).

HO-1 plasma levels were associated with bilirubin and CRP levels, disease severity scores, and the degree of organ dysfunction (V). However, the association was not very strong. The weak association of HO-1 levels with its reaction product bilirubin suggests that bilirubin is not a good marker for HO-1 activity, possibly because it is also produced by the constitutive isoform HO-2 or because the elimination of bilirubin may be impaired. The association of HO-1 with CRP was evaluated, because HO-1 levels were highest in patients with infection or inflammation. The association between HO-1 and CRP was very weak, suggesting that CRP may represent more a marker of infection and inflammation, whereas HO-1 may instead represent a marker of

oxidative stress and illness severity, because the association of HO-1 with the APACHE II and SOFA scores was stronger.

The degree of organ dysfunction was associated independently with day 1 plasma HO-1, especially the renal and ventilation failure. In addition, ICU and hospital nonsurvivors had significantly higher HO-1 plasma levels than survivors, and maximum HO-1 was associated independently with hospital mortality. HO-1 levels were increased in all critically ill patients, regardless of the diagnosis. Therefore, the higher plasma concentrations of HO-1 in more severely ill patients likely reflects the magnitude of stress of these patients and suggest a potential role for HO-1 as a general marker of disease severity. Accordingly, Chen et al. (2005) showed that HO-1 protein expression is increased in monocytes and lymphocytes of patients with coronary artery disease, and the expression levels are higher in patients with acute MI than in patients with unstable or stable angina pectoris. In addition, Scharte et al. (2006) suggested that endogenous CO production by HO-1 may reflect the disease severity. In study V, plasma HO-1 had better predictive power for ICU mortality than hospital mortality, although the maximum plasma HO-1 concentration was an independent predictor of hospital mortality. In comparison to the clinical scores, plasma HO-1 had equally good power to predict ICU mortality. However, the predictive power of HO-1 for hospital mortality, which is clinically considered more important than ICU mortality, was markedly lower than the APACHE II, SAPS II, and SOFA scores. These results support the role of HO-1 as a potential marker of disease severity and organ dysfunction and also demonstrate that HO-1 is better at predicting early mortality than long-term mortality.

Taken together, HO-1 may be useful as a general marker of illness severity. However, in acute cardiac diseases measuring HO-1 plasma levels is not likely to add value to the established cardiac markers, such as troponins I and T and N-terminal probrain natriuretic peptide (NT-proBNP). It is also noteworthy that several drugs, such as aspirin, statins, and proton pump inhibitors, increase HO-1 expression in tissues and cells and therefore they may increase plasma HO-1 as well (Grosser et al. 2003, Grosser et al. 2004, Lee et al. 2004, Becker et al. 2006). Future studies to evaluate the kinetics of plasma HO-1 and the usefulness of HO-1 as a marker of disease severity in more homogenous patient populations are warranted.

6.5 Future prospects

The present study shows that HO-1 is induced by stress in both experimental animal models and humans. The cytoprotective properties of HO-1 are well known, and the present study highlights the cardioprotective role of HO-1 and its reaction products. Despite the increasing knowledge of HO-1 in cardiovascular diseases, further studies are needed to evaluate the possible therapeutic potential of HO-1 and its reaction products in cardiovascular diseases.

The possible roles of HO-1 and especially CO in post-MI cardiac regeneration are intriguing and warrant further studies. The activation of stem/progenitor cells by CO needs to be confirmed using new CORMs as CO donors. The origin of CO-induced stem/progenitor cells should be specified, and the paracrine mechanisms leading to the activation and accumulation of stem cells should be investigated. In the present study, HO-1 induction and CO donor were used as pretreatments. In future studies, the treatments administered after infarction would be more clinically relevant. The long-term effects of HO-1 and CO treatments on cardiac regeneration shoud also be investigated.

The possible roles of HO-1 and CO in cardiac regeneration and the beneficial effects of HO-1 on cardiac cellular and extracellular remodeling open numerous therapeutic possibilities. Gene

therapy and cell therapy are new promising approaches to treatment of ischemic heart diseases. The angiogenic potential of cardiac HO-1 gene therapy has already been shown in mice (Lin et al. 2008). HO-1 overexpression has also improved survival of MSCs in infarcted rat, mouse, and swine hearts and increased production of growth factors and cytokines in MSCs (Tang et al. 2005, Zeng et al. 2008b, Jiang et al. 2010). HO-1 gene therapy could potentially limit ischemic injury by protecting the heart, and promote recovery and repair of postinfarction hearts. HO-1 gene therapy could also be used in combination with cell therapy to improve the results of cell therapy. CO mediates many of the beneficial properties of HO-1 and could be used to improve the results of cell therapy, similar to HO-1. Preconditioning of stem cells with CO could improve survival of transplanted cells in the infarcted hearts. CO may also modulate the differentiation of stem cells. In addition, systemic administration of CO as inhalation or as CORMs could promote cardiac regeneration by also activating resident CSCs in humans.

HO-1 plasma concentrations were significantly increased in all critically ill patients in study V. Being a highly inducible stress-responsive enzyme, HO-1 presumably reflects the degree of oxidative stress in different diseases. Therefore, HO-1 is a potential novel marker of illness severity, and it could also be useful in predicting the outcome in different diseases. Few studies have investigated HO-1 plasma concentrations, so the significance of plasma HO-1 in different diseases is unknown. In addition, the patient population in V was very heterogenous. Further studies in more selective patient populations are needed to evaluate the possible role of plasma HO-1 in patient management and outcome prediction.

7 CONCLUSIONS

The present study investigated the expression of HO-1 in infarcted rat hearts and the role of HO-1 in the protection against cardiac I/R injury and in the healing of postinfarction hearts. *HO-1* polymorphisms and HO-1 plasma levels were also examined in critically ill patients. The results of this study show that:

1. HO-1 expression is induced in response to MI, especially in the infarct and infarct border areas. HO-1 protein localizes in the vascular walls, the cardiomyocytes of the infarct border area, and in the monocytes/macrophages and fibroblasts of the infarct area. These results imply an important role for HO-1 in recovery of the infarcted rat hearts.

2. HO-1 induction by hemin improved the recovery of postischemic cardiac function and attenuated reperfusion-induced VF possibly by a Cx43-dependent mechanism. These results suggest that HO-1 induction potentially protects against I/R injury.

3. HO-1 and CO promote neovascularization and myocardial regeneration in the infarcted hearts by different mechanisms. CO promotes vasculogenesis and formation of new cardiomyocytes by activating c-kit+ stem/progenitor cells via HIF-1α, SDF-1α, and VEGF-B, whereas HO-1 promotes angiogenesis, possibly via SDF-1α. Thus, HO-1 and CO may provide new therapeutic strategies to enhance cardiac regeneration after MI.

4. HO-1 has beneficial effects on cellular and extracellular remodeling after MI. HO-1 protects the heart in the early phase of infarct healing by increasing survival and proliferation of cardiomyocytes. In the late phases of infarct healing, HO-1 promotes survival of cardiomyocytes, modulates the production of extracellular matrix components, and reduces perivascular fibrosis. CO mediates part of the beneficial effects of HO-1, e.g. the antiapoptotic effect, but it may also have some adverse effects, since it increased the expression of extracellular matrix components. These results suggest that HO-1 may protect against pathological ventricular remodeling.

5. HO-1 plasma levels are significantly increased in critically ill patients, including cardiac patients, and are associated with the degree of organ dysfunction and disease severity. HO-1 plasma concentrations are higher in ICU and hospital nonsurvivors than in survivors, and the maximum HO-1 concentration is associated independently with hospital mortality. Patients with the -413T/GT(L)/+99C haplotype have lower HO-1 concentrations and lower incidence of MOD. These results imply that HO-1 may be useful as a marker of disease severity, although HO-1 levels appear to be modulated by *HO-1* polymorphisms.

The present study shows that HO-1 is induced in response to stress in both experimental animal models and severely ill patients. In addition, pre-emptive HO-1 induction and CO donor pretreatment promoted post-MI cardiac regeneration, and HO-1 induction modulated post-MI cellular and extracellular remodeling. The numerous beneficial effects of the HO-1/CO pathway may provide novel therapeutic strategies to treat ischemic heart diseases in the future.

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9 REFERENCES

Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, Giordano FJ. Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. *Circulation.* 2004; 110: 3300-3305.

Adin CA, Croker BP, Agarwal A. Protective effects of exogenous bilirubin on ischemia-reperfusion injury in the isolated, perfused rat kidney. *Am J Physiol Renal Physiol.* 2005; 288: F778-84.

Agullo L, Garcia-Dorado D, Inserte J, Paniagua A, Pyrhonen P, Llevadot J, Soler-Soler J. L-arginine limits myocardial cell death secondary to hypoxia-reoxygenation by a cGMP-dependent mechanism. *Am J Physiol.* 1999; 276: H1574-80.

Aizawa T, Ishizaka N, Taguchi J, Nagai R, Mori I, Tang SS, Ingelfinger JR, Ohno M. Heme oxygenase-1 is upregulated in the kidney of angiotensin II-induced hypertensive rats : possible role in renoprotection. *Hypertension.* 2000; 35: 800-806.

Akamatsu Y, Haga M, Tyagi S, Yamashita K, Graca-Souza AV, Ollinger R, Czismadia E, May GA, Ifedigbo E, Otterbein LE, Bach FH, Soares MP. Heme oxygenase-1-derived carbon monoxide protects hearts from transplant associated ischemia reperfusion injury. *FASEB J.* 2004; 18: 771-772.

Akey J, Jin L, Xiong M. Haplotypes vs single marker linkage disequilibrium tests: what do we gain? *Eur J Hum Genet.* 2001; 9: 291-300.

Alam J, Cook JL. How many transcription factors does it take to turn on the heme oxygenase-1 gene? *Am J Respir Cell Mol Biol.* 2007; 36: 166-174.

Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem.* 1999; 274: 26071-26078.

Alam J, Camhi S, Choi AM. Identification of a second region upstream of the mouse heme oxygenase-1 gene that functions as a basal level and inducer-dependent transcription enhancer. *J Biol Chem.* 1995; 270: 11977-11984.

Alam J, Cai J, Smith A. Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5' sequences are required for induction by heme or heavy metals. *J Biol Chem.* 1994; 269: 1001-1009.

Alam J, Den Z. Distal AP-1 binding sites mediate basal level enhancement and TPA induction of the mouse heme oxygenase-1 gene. *J Biol Chem.* 1992; 267: 21894-21900.

Alam J, Shibahara S, Smith A. Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse hepatoma cells. *J Biol Chem.* 1989; 264: 6371-6375.

Alvarez-Maqueda M, El Bekay R, Alba G, Monteseirin J, Chacon P, Vega A, Martin-Nieto J, Bedoya FJ, Pintado E, Sobrino F. 15-deoxy-delta 12,14-prostaglandin J2 induces heme oxygenase-1 gene expression in a reactive oxygen species-dependent manner in human lymphocytes. *J Biol Chem.* 2004; 279: 21929-21937.

Andre L, Boissiere J, Reboul C, Perrier R, Zalvidea S, Meyer G, Thireau J, Tanguy S, Bideaux P, Hayot M, Boucher F, Obert P, Cazorla O, Richard S. Carbon monoxide pollution promotes cardiac remodeling and ventricular arrhythmia in healthy rats. *Am J Respir Crit Care Med.* 2010; 181: 587-595.

Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, Topol EJ, Penn MS. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet.* 2003; 362: 697-703.

Axelsen LN, Stahlhut M, Mohammed S, Larsen BD, Nielsen MS, Holstein-Rathlou NH, Andersen S, Jensen ON, Hennan JK, Kjolbye AL. Identification of ischemia-regulated phosphorylation sites in

connexin43: A possible target for the antiarrhythmic peptide analogue rotigaptide (ZP123). *J Mol Cell Cardiol.* 2006; 40: 790-798.

Baan C, Peeters A, Lemos F, Uitterlinden A, Doxiadis I, Claas F, Ijzermans J, Roodnat J, Weimar W. Fundamental role for HO-1 in the self-protection of renal allografts. *Am J Transplant.* 2004; 4: 811-818.

Bak I, Varadi J, Nagy N, Vecsernyes M, Tosaki A. The role of exogenous carbon monoxide in the recovery of post-ischemic cardiac function in buffer perfused isolated rat hearts. *Cell Mol Biol (Noisy-le-grand).* 2005; 51: 453-459.

Bak I, Szendrei L, Turoczi T, Papp G, Joo F, Das DK, de Leiris J, Der P, Juhasz B, Varga E, Bacskay I, Balla J, Kovacs P, Tosaki A. Heme oxygenase-1-related carbon monoxide production and ventricular fibrillation in isolated ischemic/reperfused mouse myocardium. *FASEB J.* 2003; 17: 2133-2135.

Bak I, Papp G, Turoczi T, Varga E, Szendrei L, Vecsernyes M, Joo F, Tosaki A. The role of heme oxygenase-related carbon monoxide and ventricular fibrillation in ischemic/reperfused hearts. *Free Radic Biol Med.* 2002; 33: 639-648.

Banerjee I, Fuseler JW, Price RL, Borg TK, Baudino TA. Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. *Am J Physiol Heart Circ Physiol.* 2007; 293: H1883-91.

Bao X, Reuss L, Altenberg GA. Regulation of purified and reconstituted connexin 43 hemichannels by protein kinase C-mediated phosphorylation of Serine 368. *J Biol Chem.* 2004; 279: 20058-20066.

Baranano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. *Proc Natl Acad Sci U S A.* 2002; 99: 16093-16098.

Baranano DE, Wolosker H, Bae BI, Barrow RK, Snyder SH, Ferris CD. A mammalian iron ATPase induced by iron. *J Biol Chem.* 2000; 275: 15166-15173.

Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005; 21: 263-265.

Bartunek J, Vanderheyden M, Knaapen MW, Tack W, Kockx MM, Goethals M. Deoxyribonucleic acid damage/repair proteins are elevated in the failing human myocardium due to idiopathic dilated cardiomyopathy. *J Am Coll Cardiol.* 2002; 40: 1097-103; discussion 1104-5.

Bathoorn E, Slebos DJ, Postma DS, Koeter GH, van Oosterhout AJ, van der Toorn M, Boezen HM, Kerstjens HA. Anti-inflammatory effects of inhaled carbon monoxide in patients with COPD: a pilot study. *Eur Respir J.* 2007; 30: 1131-1137.

Becker JC, Grosser N, Waltke C, Schulz S, Erdmann K, Domschke W, Schroder H, Pohle T. Beyond gastric acid reduction: proton pump inhibitors induce heme oxygenase-1 in gastric and endothelial cells. *Biochem Biophys Res Commun.* 2006; 345: 1014-1021.

Bellner L, Martinelli L, Halilovic A, Patil K, Puri N, Dunn MW, Regan RF, Schwartzman ML. Heme oxygenase-2 deletion causes endothelial cell activation marked by oxidative stress, inflammation, and angiogenesis. *J Pharmacol Exp Ther.* 2009; 331: 925-932.

Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell.* 2003; 114: 763-776.

Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P. Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med.* 2001; 344: 1750-1757.

Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. *Science.* 2009; 324: 98-102.

Boengler K, Schulz R, Heusch G. Connexin 43 signalling and cardioprotection. *Heart.* 2006; 92: 1724-1727.

Boengler K, Dodoni G, Rodriguez-Sinovas A, Cabestrero A, Ruiz-Meana M, Gres P, Konietzka I, Lopez-Iglesias C, Garcia-Dorado D, Di Lisa F, Heusch G, Schulz R. Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning. *Cardiovasc Res.* 2005; 67: 234-244.

Borger DR, Essig DA. Induction of HSP 32 gene in hypoxic cardiomyocytes is attenuated by treatment with N-acetyl-L-cysteine. *Am J Physiol.* 1998; 274: H965-73.

Botros FT, Schwartzman ML, Stier CT,Jr, Goodman AI, Abraham NG. Increase in heme oxygenase-1 levels ameliorates renovascular hypertension. *Kidney Int.* 2005; 68: 2745-2755.

Brouard S, Berberat PO, Tobiasch E, Seldon MP, Bach FH, Soares MP. Heme oxygenase-1-derived carbon monoxide requires the activation of transcription factor NF-kappa B to protect endothelial cells from tumor necrosis factor-alpha-mediated apoptosis. *J Biol Chem.* 2002; 277: 17950-17961.

Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, Soares MP. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med.* 2000; 192: 1015-1026.

Brune B, Ullrich V. Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol Pharmacol.* 1987; 32: 497-504.

Brusko TM, Wasserfall CH, Agarwal A, Kapturczak MH, Atkinson MA. An integral role for heme oxygenase-1 and carbon monoxide in maintaining peripheral tolerance by CD4+CD25+ regulatory T cells. *J Immunol.* 2005; 174: 5181-5186.

Brydun A, Watari Y, Yamamoto Y, Okuhara K, Teragawa H, Kono F, Chayama K, Oshima T, Ozono R. Reduced expression of heme oxygenase-1 in patients with coronary atherosclerosis. *Hypertens Res.* 2007; 30: 341-348.

Buis CI, van der Steege G, Visser DS, Nolte IM, Hepkema BG, Nijsten M, Slooff MJ, Porte RJ. Heme oxygenase-1 genotype of the donor is associated with graft survival after liver transplantation. *Am J Transplant.* 2008; 8: 377-385.

Bujak M, Frangogiannis NG. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. *Cardiovasc Res.* 2007; 74: 184-195.

Bukowska A, Schild L, Keilhoff G, Hirte D, Neumann M, Gardemann A, Neumann KH, Rohl FW, Huth C, Goette A, Lendeckel U. Mitochondrial dysfunction and redox signaling in atrial tachyarrhythmia. *Exp Biol Med (Maywood).* 2008; 233: 558-574.

Burger D, Xiang F, Hammoud L, Lu X, Feng Q. Role of heme oxygenase-1 in the cardioprotective effects of erythropoietin during myocardial ischemia and reperfusion. *Am J Physiol Heart Circ Physiol.* 2009; 296: H84-93.

Bussolati B, Mason JC. Dual role of VEGF-induced heme-oxygenase-1 in angiogenesis. *Antioxid Redox Signal.* 2006; 8: 1153-1163.

Bussolati B, Ahmed A, Pemberton H, Landis RC, Di Carlo F, Haskard DO, Mason JC. Bifunctional role for VEGF-induced heme oxygenase-1 in vivo: induction of angiogenesis and inhibition of leukocytic infiltration. *Blood.* 2004; 103: 761-766.

Cai CL, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, Yang L, Bu L, Liang X, Zhang X, Stallcup WB, Denton CP, McCulloch A, Chen J, Evans SM. A myocardial lineage derives from Tbx18 epicardial cells. *Nature.* 2008; 454: 104-108.

Caltabiano MM, Koestler TP, Poste G, Greig RG. Induction of 32- and 34-kDa stress proteins by sodium arsenite, heavy metals, and thiol-reactive agents. *J Biol Chem.* 1986; 261: 13381-13386.

Camelliti P, Borg TK, Kohl P. Structural and functional characterisation of cardiac fibroblasts. *Cardiovasc Res.* 2005; 65: 40-51.

Camhi SL, Alam J, Otterbein L, Sylvester SL, Choi AM. Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation. *Am J Respir Cell Mol Biol.* 1995; 13: 387-398.

Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med.* 2004; 10: 858-864.

Chen CY, Jang JH, Li MH, Surh YJ. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. *Biochem Biophys Res Commun.* 2005; 331: 993-1000.

Chen K, Maines MD. Nitric oxide induces heme oxygenase-1 via mitogen-activated protein kinases ERK and p38. *Cell Mol Biol (Noisy-le-grand).* 2000; 46: 609-617.

Chen SM, Li YG, Wang DM, Zhang GH, Tan CJ. Expression of heme oxygenase-1, hypoxia inducible factor-1alpha, and ubiquitin in peripheral inflammatory cells from patients with coronary heart disease. *Clin Chem Lab Med.* 2009; 47: 327-333.

Chen SM, Li YG, Wang DM. Study on changes of heme oxygenase-1 expression in patients with coronary heart disease. *Clin Cardiol.* 2005; 28: 197-201.

Chen X, Wilson RM, Kubo H, Berretta RM, Harris DM, Zhang X, Jaleel N, MacDonnell SM, Bearzi C, Tillmanns J, Trofimova I, Hosoda T, Mosna F, Cribbs L, Leri A, Kajstura J, Anversa P, Houser SR. Adolescent feline heart contains a population of small, proliferative ventricular myocytes with immature physiological properties. *Circ Res.* 2007; 100: 536-544.

Chen YH, Chau LY, Chen JW, Lin SJ. Serum bilirubin and ferritin levels link heme oxygenase-1 gene promoter polymorphism and susceptibility to coronary artery disease in diabetic patients. *Diabetes Care.* 2008; 31: 1615-1620.

Chen YH, Tsai HL, Chiang MT, Chau LY. Carbon monoxide-induced early thrombolysis contributes to heme oxygenase-1-mediated inhibition of neointimal growth after vascular injury in hypercholesterolemic mice. *J Biomed Sci.* 2006; 13: 721-730.

Chen YH, Chau LY, Lin MW, Chen LC, Yo MH, Chen JW, Lin SJ. Heme oxygenase-1 gene promotor microsatellite polymorphism is associated with angiographic restenosis after coronary stenting. *Eur Heart J.* 2004; 25: 39-47.

Chen YH, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, Charng MJ, Wu TC, Chen LC, Ding YA, Pan WH, Jou YS, Chau LY. Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. *Hum Genet.* 2002; 111: 1-8.

Cheng C, Noordeloos AM, Jeney V, Soares MP, Moll F, Pasterkamp G, Serruys PW, Duckers HJ. Heme oxygenase 1 determines atherosclerotic lesion progression into a vulnerable plaque. *Circulation.* 2009; 119: 3017-3027.

Cheng PY, Chen JJ, Yen MH. The expression of heme oxygenase-1 and inducible nitric oxide synthase in aorta during the development of hypertension in spontaneously hypertensive rats. *Am J Hypertens.* 2004; 17: 1127-1134.

Chin BY, Jiang G, Wegiel B, Wang HJ, Macdonald T, Zhang XC, Gallo D, Cszimadia E, Bach FH, Lee PJ, Otterbein LE. Hypoxia-inducible factor 1alpha stabilization by carbon monoxide results in cytoprotective preconditioning. *Proc Natl Acad Sci U S A.* 2007; 104: 5109-5114.

Chlopicki S, Olszanecki R, Marcinkiewicz E, Lomnicka M, Motterlini R. Carbon monoxide released by CORM-3 inhibits human platelets by a mechanism independent of soluble guanylate cyclase. *Cardiovasc Res.* 2006; 71: 393-401.

Choi BM, Pae HO, Jeong YR, Oh GS, Jun CD, Kim BR, Kim YM, Chung HT. Overexpression of heme oxygenase (HO)-1 renders Jurkat T cells resistant to fas-mediated apoptosis: involvement of iron released by HO-1. *Free Radic Biol Med.* 2004; 36: 858-871.

Cisowski J, Loboda A, Jozkowicz A, Chen S, Agarwal A, Dulak J. Role of heme oxygenase-1 in hydrogen peroxide-induced VEGF synthesis: effect of HO-1 knockout. *Biochem Biophys Res Commun.* 2005; 326: 670-676.

Clark JE, Foresti R, Green CJ, Motterlini R. Dynamics of haem oxygenase-1 expression and bilirubin production in cellular protection against oxidative stress. *Biochem J.* 2000a; 348 Pt 3: 615-619.

Clark JE, Foresti R, Sarathchandra P, Kaur H, Green CJ, Motterlini R. Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. *Am J Physiol Heart Circ Physiol.* 2000b; 278: H643-51.

Cobb MH. MAP kinase pathways. *Prog Biophys Mol Biol.* 1999; 71: 479-500.

Converso DP, Taille C, Carreras MC, Jaitovich A, Poderoso JJ, Boczkowski J. HO-1 is located in liver mitochondria and modulates mitochondrial heme content and metabolism. *FASEB J.* 2006; 20: 1236-1238.

Corradi D, Callegari S, Maestri R, Benussi S, Bosio S, De Palma G, Alinovi R, Caglieri A, Goldoni M, Mozzoni P, Pastori P, Manotti L, Nascimbene S, Dorigo E, Rusconi R, Astorri E, Alfieri O. Heme oxygenase-1 expression in the left atrial myocardium of patients with chronic atrial fibrillation related to mitral valve disease: its regional relationship with structural remodeling. *Hum Pathol.* 2008; 39: 1162-1171.

Courtney AE, McNamee PT, Middleton D, Heggarty S, Patterson CC, Maxwell AP. Association of functional heme oxygenase-1 gene promoter polymorphism with renal transplantation outcomes. *Am J Transplant.* 2007; 7: 908-913.

Crow DS, Beyer EC, Paul DL, Kobe SS, Lau AF. Phosphorylation of connexin43 gap junction protein in uninfected and Rous sarcoma virus-transformed mammalian fibroblasts. *Mol Cell Biol.* 1990; 10: 1754-1763.

Cruse I, Maines MD. Evidence suggesting that the two forms of heme oxygenase are products of different genes. *J Biol Chem.* 1988; 263: 3348-3353.

Csonka C, Szilvassy Z, Fulop F, Pali T, Blasig IE, Tosaki A, Schulz R, Ferdinandy P. Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. *Circulation.* 1999; 100: 2260-2266.

Cudmore M, Ahmad S, Al-Ani B, Fujisawa T, Coxall H, Chudasama K, Devey LR, Wigmore SJ, Abbas A, Hewett PW, Ahmed A. Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1. *Circulation.* 2007; 115: 1789-1797.

Czibik G, Sagave J, Martinov V, Ishaq B, Sohl M, Sefland I, Carlsen H, Farnebo F, Blomhoff R, Valen G. Cardioprotection by hypoxia-inducible factor 1 alpha transfection in skeletal muscle is dependent on haem oxygenase activity in mice. *Cardiovasc Res.* 2009; 82: 107-114.

Dahms TE, Younis LT, Wiens RD, Zarnegar S, Byers SL, Chaitman BR. Effects of carbon monoxide exposure in patients with documented cardiac arrhythmias. *J Am Coll Cardiol.* 1993; 21: 442-450.

Dallas ML, Scragg JL, Peers C. Inhibition of L-type Ca(2+) channels by carbon monoxide. *Adv Exp Med Biol.* 2009; 648: 89-95.

Dawn B, Stein AB, Urbanek K, Rota M, Whang B, Rastaldo R, Torella D, Tang XL, Rezazadeh A, Kajstura J, Leri A, Hunt G, Varma J, Prabhu SD, Anversa P, Bolli R. Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. *Proc Natl Acad Sci U S A.* 2005; 102: 3766-3771.

De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res.* 1998; 82: 1094-1101.

De Mello WC. Atrial natriuretic factor reduces cell coupling in the failing heart, an effect mediated by cyclic GMP. *J Cardiovasc Pharmacol.* 1998; 32: 75-79.

Denschlag D, Marculescu R, Unfried G, Hefler LA, Exner M, Hashemi A, Riener EK, Keck C, Tempfer CB, Wagner O. The size of a microsatellite polymorphism of the haem oxygenase 1 gene is associated with idiopathic recurrent miscarriage. *Mol Hum Reprod.* 2004; 10: 211-214.

Deramaudt BM, Braunstein S, Remy P, Abraham NG. Gene transfer of human heme oxygenase into coronary endothelial cells potentially promotes angiogenesis. *J Cell Biochem.* 1998; 68: 121-127.

Deramaudt TB, da Silva JL, Remy P, Kappas A, Abraham NG. Negative regulation of human heme oxygenase in microvessel endothelial cells by dexamethasone. *Proc Soc Exp Biol Med.* 1999; 222: 185-193.

Deshane J, Chen S, Caballero S, Grochot-Przeczek A, Was H, Li Calzi S, Lach R, Hock TD, Chen B, Hill-Kapturczak N, Siegal GP, Dulak J, Jozkowicz A, Grant MB, Agarwal A. Stromal cell-derived factor 1 promotes angiogenesis via a heme oxygenase 1-dependent mechanism. *J Exp Med.* 2007; 204: 605-618.

Dick P, Schillinger M, Minar E, Mlekusch W, Amighi J, Sabeti S, Schlager O, Raith M, Endler G, Mannhalter C, Wagner O, Exner M. Haem oxygenase-1 genotype and cardiovascular adverse events in patients with peripheral artery disease. *Eur J Clin Invest.* 2005; 35: 731-737.

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A.* 2002; 99: 11908-11913.

Dolan MC. Carbon monoxide poisoning. *CMAJ.* 1985; 133: 392-399.

Drummond GS, Kappas A. Chemoprevention of neonatal jaundice: potency of tin-protoporphyrin in an animal model. *Science.* 1982; 217: 1250-1252.

Drummond GS, Kappas A. Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation. *Proc Natl Acad Sci U S A.* 1981; 78: 6466-6470.

Duckers HJ, Boehm M, True AL, Yet SF, San H, Park JL, Clinton Webb R, Lee ME, Nabel GJ, Nabel EG. Heme oxygenase-1 protects against vascular constriction and proliferation. *Nat Med.* 2001; 7: 693-698.

Dulak J, Deshane J, Jozkowicz A, Agarwal A. Heme oxygenase-1 and carbon monoxide in vascular pathobiology: focus on angiogenesis. *Circulation.* 2008; 117: 231-241.

Dulak J, Jozkowicz A, Foresti R, Kasza A, Frick M, Huk I, Green CJ, Pachinger O, Weidinger F, Motterlini R. Heme oxygenase activity modulates vascular endothelial growth factor synthesis in vascular smooth muscle cells. *Antioxid Redox Signal.* 2002; 4: 229-240.

Durante W, Peyton KJ, Schafer AI. Platelet-derived growth factor stimulates heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1999; 19: 2666-2672.

Durante W, Kroll MH, Christodoulides N, Peyton KJ, Schafer AI. Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ Res.* 1997; 80: 557-564.

Eide IP, Isaksen CV, Salvesen KA, Langaas M, Schonberg SA, Austgulen R. Decidual expression and maternal serum levels of heme oxygenase 1 are increased in pre-eclampsia. *Acta Obstet Gynecol Scand.* 2008; 87: 272-279.

Elbirt KK, Whitmarsh AJ, Davis RJ, Bonkovsky HL. Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. *J Biol Chem.* 1998; 273: 8922-8931.

Elmadbouh I, Haider HK, Jiang S, Idris NM, Lu G, Ashraf M. Ex vivo delivered stromal cell-derived factor-1alpha promotes stem cell homing and induces angiomyogenesis in the infarcted myocardium. *J Mol Cell Cardiol.* 2007; 42: 792-803.

Emerit J, Beaumont C, Trivin F. Iron metabolism, free radicals, and oxidative injury. *Biomed Pharmacother.* 2001; 55: 333-339.

Endler G, Exner M, Schillinger M, Marculescu R, Sunder-Plassmann R, Raith M, Jordanova N, Wojta J, Mannhalter C, Wagner OF, Huber K. A microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with increased bilirubin and HDL levels but not with coronary artery disease. *Thromb Haemost.* 2004; 91: 155-161.

Engel FB, Hsieh PC, Lee RT, Keating MT. FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. *Proc Natl Acad Sci U S A.* 2006; 103: 15546-15551.

Exner M, Bohmig GA, Schillinger M, Regele H, Watschinger B, Horl WH, Raith M, Mannhalter C, Wagner OF. Donor heme oxygenase-1 genotype is associated with renal allograft function. *Transplantation.* 2004; 77: 538-542.

Faleo G, Neto JS, Kohmoto J, Tomiyama K, Shimizu H, Takahashi T, Wang Y, Sugimoto R, Choi AM, Stolz DB, Carrieri G, McCurry KR, Murase N, Nakao A. Carbon monoxide ameliorates renal cold ischemia-reperfusion injury with an upregulation of vascular endothelial growth factor by activation of hypoxia-inducible factor. *Transplantation.* 2008; 85: 1833-1840.

Ferdinandy P, Das DK, Tosaki A. Pacing-induced ventricular fibrillation leading to oxygen free radical formation in aerobically perfused rat hearts. *J Mol Cell Cardiol.* 1993; 25: 683-692.

Ferenbach DA, Ramdas V, Spencer N, Marson L, Anegon I, Hughes J, Kluth DC. Macrophages Expressing Heme Oxygenase-1 Improve Renal Function in Ischemia/Reperfusion Injury. *Mol Ther.* 2010; .

Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD, Snyder SH. Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nat Cell Biol.* 1999; 1: 152-157.

Fondevila C, Shen XD, Tsuchiyashi S, Yamashita K, Csizmadia E, Lassman C, Busuttil RW, Kupiec-Weglinski JW, Bach FH. Biliverdin therapy protects rat livers from ischemia and reperfusion injury. *Hepatology.* 2004; 40: 1333-1341.

Foo RS, Siow RC, Brown MJ, Bennett MR. Heme oxygenase-1 gene transfer inhibits angiotensin II-mediated rat cardiac myocyte apoptosis but not hypertrophy. *J Cell Physiol.* 2006; 209: 1-7.

Foresti R, Hammad J, Clark JE, Johnson TR, Mann BE, Friebe A, Green CJ, Motterlini R. Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. *Br J Pharmacol.* 2004; 142: 453-460.

Foresti R, Goatly H, Green CJ, Motterlini R. Role of heme oxygenase-1 in hypoxia-reoxygenation: requirement of substrate heme to promote cardioprotection. *Am J Physiol Heart Circ Physiol.* 2001; 281: H1976-84.

Foresti R, Sarathchandra P, Clark JE, Green CJ, Motterlini R. Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. *Biochem J.* 1999; 339 (Pt 3): 729-736.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol.* 1996; 16: 4604-4613.

Francis GS, Anwar F, Bank AJ, Kubo SH, Jessurun J. Apoptosis, Bcl-2, and proliferating cell nuclear antigen in the failing human heart: observations made after implantation of left ventricular assist device. *J Card Fail.* 1999; 5: 308-315.

Frangogiannis NG, Ren G, Dewald O, Zymek P, Haudek S, Koerting A, Winkelmann K, Michael LH, Lawler J, Entman ML. Critical role of endogenous thrombospondin-1 in preventing expansion of healing myocardial infarcts. *Circulation.* 2005; 111: 2935-2942.

Fransioli J, Bailey B, Gude NA, Cottage CT, Muraski JA, Emmanuel G, Wu W, Alvarez R, Rubio M, Ottolenghi S, Schaefer E, Sussman MA. Evolution of the c-kit-positive cell response to pathological challenge in the myocardium. *Stem Cells.* 2008; 26: 1315-1324.

Fujita T, Toda K, Karimova A, Yan SF, Naka Y, Yet SF, Pinsky DJ. Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. *Nat Med.* 2001; 7: 598-604.

Fukano Y, Oishi M, Chibana F, Numazawa S, Yoshida T. Analysis of the expression of heme oxygenase-1 gene in human alveolar epithelial cells exposed to cigarette smoke condensate. *J Toxicol Sci.* 2006; 31: 99-109.

Fukuda Y, Sassa S. Effect of interleukin-11 on the levels of mRNAs encoding heme oxygenase and haptoglobin in human HepG2 hepatoma cells. *Biochem Biophys Res Commun.* 1993; 193: 297-302.

Furchgott RF, Jothianandan D. Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels.* 1991; 28: 52-61.

Gaedeke J, Noble NA, Border WA. Curcumin blocks fibrosis in anti-Thy 1 glomerulonephritis through up-regulation of heme oxygenase 1. *Kidney Int.* 2005; 68: 2042-2049.

Garcia-Dorado D, Rodriguez-Sinovas A, Ruiz-Meana M. Gap junction-mediated spread of cell injury and death during myocardial ischemia-reperfusion. *Cardiovasc Res.* 2004; 61: 386-401.

Garcia-Dorado D, Inserte J, Ruiz-Meana M, Gonzalez MA, Solares J, Julia M, Barrabes JA, Soler-Soler J. Gap junction uncoupler heptanol prevents cell-to-cell progression of hypercontracture and limits necrosis during myocardial reperfusion. *Circulation.* 1997; 96: 3579-3586.

Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res.* 2008; 103: 1204-1219.

Grabellus F, Schmid C, Levkau B, Breukelmann D, Halloran PF, August C, Takeda N, Takeda A, Wilhelm M, Deng MC, Baba HA. Reduction of hypoxia-inducible heme oxygenase-1 in the myocardium after left ventricular mechanical support. *J Pathol.* 2002; 197: 230-237.

Granato A, Gores G, Vilei MT, Tolando R, Ferraresso C, Muraca M. Bilirubin inhibits bile acid induced apoptosis in rat hepatocytes. *Gut.* 2003; 52: 1774-1778.

Grosser N, Hemmerle A, Berndt G, Erdmann K, Hinkelmann U, Schurger S, Wijayanti N, Immenschuh S, Schroder H. The antioxidant defense protein heme oxygenase 1 is a novel target for statins in endothelial cells. *Free Radic Biol Med.* 2004; 37: 2064-2071.

Grosser N, Abate A, Oberle S, Vreman HJ, Dennery PA, Becker JC, Pohle T, Seidman DS, Schroder H. Heme oxygenase-1 induction may explain the antioxidant profile of aspirin. *Biochem Biophys Res Commun.* 2003; 308: 956-960.

Guenegou A, Leynaert B, Benessiano J, Pin I, Demoly P, Neukirch F, Boczkowski J, Aubier M. Association of lung function decline with the heme oxygenase-1 gene promoter microsatellite polymorphism in a general population sample. Results from the European Community Respiratory Health Survey (ECRHS), France. *J Med Genet.* 2006; 43: e43.

Gulesserian T, Wenzel C, Endler G, Sunder-Plassmann R, Marsik C, Mannhalter C, Iordanova N, Gyongyosi M, Wojta J, Mustafa S, Wagner O, Huber K. Clinical restenosis after coronary stent implantation is associated with the heme oxygenase-1 gene promoter polymorphism and the heme oxygenase-1 +99G/C variant. *Clin Chem.* 2005; 51: 1661-1665.

Guo Y, Stein AB, Wu WJ, Tan W, Zhu X, Li QH, Dawn B, Motterlini R, Bolli R. Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. *Am J Physiol Heart Circ Physiol.* 2004; 286: H1649-53.

Hagberg CE, Falkevall A, Wang X, Larsson E, Huusko J, Nilsson I, van Meeteren LA, Samen E, Lu L, Vanwildemeersch M, Klar J, Genove G, Pietras K, Stone-Elander S, Claesson-Welsh L, Yla-Herttuala S, Lindahl P, Eriksson U. Vascular endothelial growth factor B controls endothelial fatty acid uptake. *Nature.* 2010; 464: 917-921.

Hangaishi M, Ishizaka N, Aizawa T, Kurihara Y, Taguchi J, Nagai R, Kimura S, Ohno M. Induction of heme oxygenase-1 can act protectively against cardiac ischemia/reperfusion in vivo. *Biochem Biophys Res Commun.* 2000; 279: 582-588.

Hartsfield CL, Alam J, Cook JL, Choi AM. Regulation of heme oxygenase-1 gene expression in vascular smooth muscle cells by nitric oxide. *Am J Physiol.* 1997; 273: L980-8.

Hayashi S, Omata Y, Sakamoto H, Higashimoto Y, Hara T, Sagara Y, Noguchi M. Characterization of rat heme oxygenase-3 gene. Implication of processed pseudogenes derived from heme oxygenase-2 gene. *Gene.* 2004; 336: 241-250.

Hayashi S, Takamiya R, Yamaguchi T, Matsumoto K, Tojo SJ, Tamatani T, Kitajima M, Makino N, Ishimura Y, Suematsu M. Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme. *Circ Res.* 1999; 85: 663-671.

Hegazi RA, Rao KN, Mayle A, Sepulveda AR, Otterbein LE, Plevy SE. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. *J Exp Med.* 2005; 202: 1703-1713.

Hinderliter AL, Adams KF,Jr, Price CJ, Herbst MC, Koch G, Sheps DS. Effects of low-level carbon monoxide exposure on resting and exercise-induced ventricular arrhythmias in patients with coronary artery disease and no baseline ectopy. *Arch Environ Health.* 1989; 44: 89-93.

Hirai H, Kubo H, Yamaya M, Nakayama K, Numasaki M, Kobayashi S, Suzuki S, Shibahara S, Sasaki H. Microsatellite polymorphism in heme oxygenase-1 gene promoter is associated with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines. *Blood.* 2003; 102: 1619-1621.

Holweg CT, Balk AH, Uitterlinden AG, Niesters HG, Maat LP, Weimar W, Baan CC. Functional heme oxygenase-1 promoter polymorphism in relation to heart failure and cardiac transplantation. *J Heart Lung Transplant.* 2005; 24: 493-497.

Hopkins PN, Wu LL, Hunt SC, James BC, Vincent GM, Williams RR. Higher serum bilirubin is associated with decreased risk for early familial coronary artery disease. *Arterioscler Thromb Vasc Biol.* 1996; 16: 250-255.

Horvath SM, Raven PB, Dahms TE, Gray DJ. Maximal aerobic capacity at different levels of carboxyhemoglobin. *J Appl Physiol.* 1975; 38: 300-303.

Hu CM, Chen YH, Chiang MT, Chau LY. Heme oxygenase-1 inhibits angiotensin II-induced cardiac hypertrophy in vitro and in vivo. *Circulation.* 2004; 110: 309-316.

Hu X, Dai S, Wu WJ, Tan W, Zhu X, Mu J, Guo Y, Bolli R, Rokosh G. Stromal cell derived factor-1 alpha confers protection against myocardial ischemia/reperfusion injury: role of the cardiac stromal cell derived factor-1 alpha CXCR4 axis. *Circulation.* 2007; 116: 654-663.

Hund TJ, Lerner DL, Yamada KA, Schuessler RB, Saffitz JE. Protein kinase Cepsilon mediates salutary effects on electrical coupling induced by ischemic preconditioning. *Heart Rhythm.* 2007; 4: 1183-1193.

Hussain AS, Marks GS, Brien JF, Nakatsu K. The soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ) inhibits relaxation of rabbit aortic rings induced by carbon monoxide, nitric oxide, and glyceryl trinitrate. *Can J Physiol Pharmacol.* 1997; 75: 1034-1037.

Hyvelin JM, Maurel B, Uzbekov R, Motterlini R, Lermusiaux P. Hemin prevents in-stent stenosis in rat and rabbit models by inducing heme-oxygenase-1. *J Vasc Surg.* 2010; 51: 417-428.

Idriss NK, Blann AD, Lip GY. Hemoxygenase-1 in cardiovascular disease. *J Am Coll Cardiol.* 2008; 52: 971-978.

Imai T, Morita T, Shindo T, Nagai R, Yazaki Y, Kurihara H, Suematsu M, Katayama S. Vascular smooth muscle cell-directed overexpression of heme oxygenase-1 elevates blood pressure through attenuation of nitric oxide-induced vasodilation in mice. *Circ Res.* 2001; 89: 55-62.

Immenschuh S, Shan Y, Kroll H, Santoso S, Wossmann W, Bein G, Bonkovsky HL. Marked hyperbilirubinemia associated with the heme oxygenase-1 gene promoter microsatellite polymorphism in a boy with autoimmune hemolytic anemia. *Pediatrics.* 2007; 119: e764-7.

Ishikawa K, Kimura S, Kobayashi A, Sato T, Matsumoto H, Ujiie Y, Nakazato K, Mitsugi M, Maruyama Y. Increased reactive oxygen species and anti-oxidative response in mitochondrial cardiomyopathy. *Circ J.* 2005; 69: 617-620.

Ishikawa K, Sugawara D, Goto J, Watanabe Y, Kawamura K, Shiomi M, Itabe H, Maruyama Y. Heme oxygenase-1 inhibits atherogenesis in Watanabe heritable hyperlipidemic rabbits. *Circulation.* 2001a; 104: 1831-1836.

Ishikawa K, Sugawara D, Wang X, Suzuki K, Itabe H, Maruyama Y, Lusis AJ. Heme oxygenase-1 inhibits atherosclerotic lesion formation in ldl-receptor knockout mice. *Circ Res.* 2001b; 88: 506-512.

Ishikawa K, Navab M, Leitinger N, Fogelman AM, Lusis AJ. Induction of heme oxygenase-1 inhibits the monocyte transmigration induced by mildly oxidized LDL. *J Clin Invest.* 1997; 100: 1209-1216.

Ishikawa K, Sato M, Yoshida T. Expression of rat heme oxygenase in Escherichia coli as a catalytically active, full-length form that binds to bacterial membranes. *Eur J Biochem.* 1991; 202: 161-165.

Ishizaka N, Aizawa T, Mori I, Taguchi J, Yazaki Y, Nagai R, Ohno M. Heme oxygenase-1 is upregulated in the rat heart in response to chronic administration of angiotensin II. *Am J Physiol Heart Circ Physiol.* 2000; 279: H672-8.

Ishizaka N, Griendling KK. Heme oxygenase-1 is regulated by angiotensin II in rat vascular smooth muscle cells. *Hypertension.* 1997; 29: 790-795.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T, Yamamoto M. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells.* 2003; 8: 379-391.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 1999; 13: 76-86.

Jadhav A, Ndisang JF. Heme arginate suppresses cardiac lesions and hypertrophy in deoxycorticosterone acetate-salt hypertension. *Exp Biol Med (Maywood).* 2009; 234: 764-778.

Jaggar JH, Leffler CW, Cheranov SY, Tcheranova D, E S, Cheng X. Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca2+ sparks to Ca2+-activated K+ channels. *Circ Res.* 2002; 91: 610-617.

Jansen T, Hortmann M, Oelze M, Opitz B, Steven S, Schell R, Knorr M, Karbach S, Schuhmacher S, Wenzel P, Munzel T, Daiber A. Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection by heme oxygenase-1-evidence for direct and indirect antioxidant actions of bilirubin. *J Mol Cell Cardiol.* 2010; 49: 186-195.

Jazwa A, Loboda A, Golda S, Cisowski J, Szelag M, Zagorska A, Sroczynska P, Drukala J, Jozkowicz A, Dulak J. Effect of heme and heme oxygenase-1 on vascular endothelial growth factor synthesis and angiogenic potency of human keratinocytes. *Free Radic Biol Med.* 2006; 40: 1250-1263.

Jiang Y, Chen L, Tang Y, Ma G, Shen C, Qi C, Zhu Q, Yao Y, Liu N. HO-1 gene overexpression enhances the beneficial effects of superparamagnetic iron oxide labeled bone marrow stromal cells transplantation in swine hearts underwent ischemia/reperfusion: an MRI study. *Basic Res Cardiol.* 2010; 105: 431-442.

Jin Y, Choi AM. Cytoprotection of heme oxygenase-1/carbon monoxide in lung injury. *Proc Am Thorac Soc.* 2005; 2: 232-235.

Johns DG, Zelent D, Ao Z, Bradley BT, Cooke A, Contino L, Hu E, Douglas SA, Jaye MC. Heme-oxygenase induction inhibits arteriolar thrombosis in vivo: effect of the non-substrate inducer cobalt protoporphyrin. *Eur J Pharmacol.* 2009; 606: 109-114.

Johnson FK, Durante W, Peyton KJ, Johnson RA. Heme oxygenase-mediated endothelial dysfunction in DOCA-salt, but not in spontaneously hypertensive, rat arterioles. *Am J Physiol Heart Circ Physiol.* 2004; 286: H1681-7.

Johnson FK, Durante W, Peyton KJ, Johnson RA. Heme oxygenase inhibitor restores arteriolar nitric oxide function in dahl rats. *Hypertension.* 2003; 41: 149-155.

Johnson FK, Johnson RA. Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles. *Am J Physiol Regul Integr Comp Physiol.* 2003; 285: R536-41.

Johnson FK, Teran FJ, Prieto-Carrasquero M, Johnson RA. Vascular effects of a heme oxygenase inhibitor are enhanced in the absence of nitric oxide. *Am J Hypertens.* 2002; 15: 1074-1080.

Johnson RA, Teran FJ, Durante W, Peyton KJ, Johnson FK. Enhanced heme oxygenase-mediated coronary vasodilation in Dahl salt-sensitive hypertension. *Am J Hypertens.* 2004; 17: 25-30.

Johnson RA, Lavesa M, DeSeyn K, Scholer MJ, Nasjletti A. Heme oxygenase substrates acutely lower blood pressure in hypertensive rats. *Am J Physiol.* 1996; 271: H1132-8.

Jozkowicz A, Huk I, Nigisch A, Weigel G, Dietrich W, Motterlini R, Dulak J. Heme oxygenase and angiogenic activity of endothelial cells: stimulation by carbon monoxide and inhibition by tin protoporphyrin-IX. *Antioxid Redox Signal.* 2003; 5: 155-162.

Jozkowicz A, Huk I, Nigisch A, Weigel G, Weidinger F, Dulak J. Effect of prostaglandin-J(2) on VEGF synthesis depends on the induction of heme oxygenase-1. *Antioxid Redox Signal.* 2002; 4: 577-585.

Juan SH, Lee TS, Tseng KW, Liou JY, Shyue SK, Wu KK, Chau LY. Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice. *Circulation.* 2001; 104: 1519-1525.

Jyrkkänen HK, Kansanen E, Inkala M, Kivelä AM, Hurttila H, Heinonen SE, Goldsteins G, Jauhiainen S, Tiainen S, Makkonen H, Oskolkova O, Afonyushkin T, Koistinaho J, Yamamoto M, Bochkov VN, Ylä-Herttuala S, Levonen AL. Nrf2 regulates antioxidant gene expression evoked by oxidized phospholipids in endothelial cells and murine arteries in vivo. *Circ Res.* 2008; 103: e1-9.

Kacimi R, Chentoufi J, Honbo N, Long CS, Karliner JS. Hypoxia differentially regulates stress proteins in cultured cardiomyocytes: role of the p38 stress-activated kinase signaling cascade, and relation to cytoprotection. *Cardiovasc Res.* 2000; 46: 139-150.

Kaga S, Zhan L, Matsumoto M, Maulik N. Resveratrol enhances neovascularization in the infarcted rat myocardium through the induction of thioredoxin-1, heme oxygenase-1 and vascular endothelial growth factor. *J Mol Cell Cardiol.* 2005; 39: 813-822.

Kairisto V, Poola A. Software for illustrative presentation of basic clinical characteristics of laboratory tests--GraphROC for Windows. *Scand J Clin Lab Invest Suppl.* 1995; 222: 43-60.

Kajstura J, Urbanek K, Perl S, Hosoda T, Zheng H, Ogorek B, Ferreira-Martins J, Goichberg P, Rondon-Clavo C, Sanada F, D'Amario D, Rota M, Del Monte F, Orlic D, Tisdale J, Leri A, Anversa P. Cardiomyogenesis in the adult human heart. *Circ Res.* 2010; 107: 305-315.

Kaneda H, Ohno M, Taguchi J, Togo M, Hashimoto H, Ogasawara K, Aizawa T, Ishizaka N, Nagai R. Heme oxygenase-1 gene promoter polymorphism is associated with coronary artery disease in Japanese patients with coronary risk factors. *Arterioscler Thromb Vasc Biol.* 2002; 22: 1680-1685.

Katori M, Anselmo DM, Busuttil RW, Kupiec-Weglinski JW. A novel strategy against ischemia and reperfusion injury: cytoprotection with heme oxygenase system. *Transpl Immunol.* 2002a; 9: 227-233.

Katori M, Buelow R, Ke B, Ma J, Coito AJ, Iyer S, Southard D, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 overexpression protects rat hearts from cold ischemia/reperfusion injury via an antiapoptotic pathway. *Transplantation.* 2002b; 73: 287-292.

Kaur H, Hughes MN, Green CJ, Naughton P, Foresti R, Motterlini R. Interaction of bilirubin and biliverdin with reactive nitrogen species. *FEBS Lett.* 2003; 543: 113-119.

Kawamura K, Ishikawa K, Wada Y, Kimura S, Matsumoto H, Kohro T, Itabe H, Kodama T, Maruyama Y. Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction. *Arterioscler Thromb Vasc Biol.* 2005; 25: 155-160.

Kawashima A, Oda Y, Yachie A, Koizumi S, Nakanishi I. Heme oxygenase-1 deficiency: the first autopsy case. *Hum Pathol.* 2002; 33: 125-130.

Kelly BD, Hackett SF, Hirota K, Oshima Y, Cai Z, Berg-Dixon S, Rowan A, Yan Z, Campochiaro PA, Semenza GL. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. *Circ Res.* 2003; 93: 1074-1081.

Keyse SM, Emslie EA. Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature.* 1992; 359: 644-647.

Keyse SM, Applegate LA, Tromvoukis Y, Tyrrell RM. Oxidant stress leads to transcriptional activation of the human heme oxygenase gene in cultured skin fibroblasts. *Mol Cell Biol.* 1990; 10: 4967-4969.

Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci U S A.* 1989; 86: 99-103.

Keyse SM, Tyrrell RM. Both near ultraviolet radiation and the oxidizing agent hydrogen peroxide induce a 32-kDa stress protein in normal human skin fibroblasts. *J Biol Chem.* 1987; 262: 14821-14825.

Kharitonov VG, Sharma VS, Pilz RB, Magde D, Koesling D. Basis of guanylate cyclase activation by carbon monoxide. *Proc Natl Acad Sci U S A.* 1995; 92: 2568-2571.

Kim EH, Na HK, Surh YJ. Upregulation of VEGF by 15-deoxy-Delta12,14-prostaglandin J2 via heme oxygenase-1 and ERK1/2 signaling in MCF-7 cells. *Ann N Y Acad Sci.* 2006; 1090: 375-384.

Kim HP, Wang X, Galbiati F, Ryter SW, Choi AM. Caveolae compartmentalization of heme oxygenase-1 in endothelial cells. *FASEB J.* 2004; 18: 1080-1089.

Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. *Crit Care Med.* 1985; 13: 818-829.

Kutty RK, Kutty G, Rodriguez IR, Chader GJ, Wiggert B. Chromosomal localization of the human heme oxygenase genes: heme oxygenase-1 (HMOX1) maps to chromosome 22q12 and heme oxygenase-2 (HMOX2) maps to chromosome 16p13.3. *Genomics.* 1994; 20: 513-516.

Lähteenvuo JE, Lähteenvuo MT, Kivelä A, Rosenlew C, Falkevall A, Klar J, Heikura T, Rissanen TT, Vähakangas E, Korpisalo P, Enholm B, Carmeliet P, Alitalo K, Eriksson U, Ylä-Herttuala S. Vascular endothelial growth factor-B induces myocardium-specific angiogenesis and arteriogenesis via vascular endothelial growth factor receptor-1- and neuropilin receptor-1-dependent mechanisms. *Circulation.* 2009; 119: 845-856.

Lampe PD, Cooper CD, King TJ, Burt JM. Analysis of Connexin43 phosphorylated at S325, S328 and S330 in normoxic and ischemic heart. *J Cell Sci.* 2006; 119: 3435-3442.

Lampe PD, TenBroek EM, Burt JM, Kurata WE, Johnson RG, Lau AF. Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J Cell Biol.* 2000; 149: 1503-1512.

Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, Abraham NG. Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proc Natl Acad Sci U S A.* 1994; 91: 5987-5991.

Le Gall JR, Lemeshow S, Saulnier F. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. *JAMA.* 1993; 270: 2957-2963.

Lee PJ, Camhi SL, Chin BY, Alam J, Choi AM. AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279: L175-82.

Lee PJ, Jiang BH, Chin BY, Iyer NV, Alam J, Semenza GL, Choi AM. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J Biol Chem.* 1997; 272: 5375-5381.

Lee SS, Gao W, Mazzola S, Thomas MN, Csizmadia E, Otterbein LE, Bach FH, Wang H. Heme oxygenase-1, carbon monoxide, and bilirubin induce tolerance in recipients toward islet allografts by modulating T regulatory cells. *FASEB J.* 2007; 21: 3450-3457.

Lee TS, Chang CC, Zhu Y, Shyy JY. Simvastatin induces heme oxygenase-1: a novel mechanism of vessel protection. *Circulation.* 2004; 110: 1296-1302.

Lee TS, Tsai HL, Chau LY. Induction of heme oxygenase-1 expression in murine macrophages is essential for the anti-inflammatory effect of low dose 15-deoxy-Delta 12,14-prostaglandin J2. *J Biol Chem.* 2003; 278: 19325-19330.

Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med.* 2002; 8: 240-246.

Lee TS, Shiao MS, Pan CC, Chau LY. Iron-deficient diet reduces atherosclerotic lesions in apoE-deficient mice. *Circulation.* 1999; 99: 1222-1229.

Lerner DL, Yamada KA, Schuessler RB, Saffitz JE. Accelerated onset and increased incidence of ventricular arrhythmias induced by ischemia in Cx43-deficient mice. *Circulation.* 2000; 101: 547-552.

Levere RD, Martasek P, Escalante B, Schwartzman ML, Abraham NG. Effect of heme arginate administration on blood pressure in spontaneously hypertensive rats. *J Clin Invest.* 1990; 86: 213-219.

Levonen AL, Inkala M, Heikura T, Jauhiainen S, Jyrkkänen HK, Kansanen E, Määttä K, Romppanen E, Turunen P, Rutanen J, Ylä-Herttuala S. Nrf2 gene transfer induces antioxidant enzymes and suppresses smooth muscle cell growth in vitro and reduces oxidative stress in rabbit aorta in vivo. *Arterioscler Thromb Vasc Biol.* 2007; 27: 741-747.

Li Volti G, Sacerdoti D, Sangras B, Vanella A, Mezentsev A, Scapagnini G, Falck JR, Abraham NG. Carbon monoxide signaling in promoting angiogenesis in human microvessel endothelial cells. *Antioxid Redox Signal.* 2005; 7: 704-710.

Li Volti G, Wang J, Traganos F, Kappas A, Abraham NG. Differential effect of heme oxygenase-1 in endothelial and smooth muscle cell cycle progression. *Biochem Biophys Res Commun.* 2002; 296: 1077-1082.

Li Q, Guo Y, Ou Q, Cui C, Wu WJ, Tan W, Zhu X, Lanceta LB, Sanganalmath SK, Dawn B, Shinmura K, Rokosh GD, Wang S, Bolli R. Gene transfer of inducible nitric oxide synthase affords cardioprotection by upregulating heme oxygenase-1 via a nuclear factor-{kappa}B-dependent pathway. *Circulation.* 2009; 120: 1222-1230.

Li X, Tjwa M, Van Hove I, Enholm B, Neven E, Paavonen K, Jeltsch M, Juan TD, Sievers RE, Chorianopoulos E, Wada H, Vanwildemeersch M, Noel A, Foidart JM, Springer ML, von Degenfeld G, Dewerchin M, Blau HM, Alitalo K, Eriksson U, Carmeliet P, Moons L. Reevaluation of the role of VEGF-B suggests a restricted role in the revascularization of the ischemic myocardium. *Arterioscler Thromb Vasc Biol.* 2008; 28: 1614-1620.

Li X, Heinzel FR, Boengler K, Schulz R, Heusch G. Role of connexin 43 in ischemic preconditioning does not involve intercellular communication through gap junctions. *J Mol Cell Cardiol.* 2004; 36: 161-163.

Limana F, Zacheo A, Mocini D, Mangoni A, Borsellino G, Diamantini A, De Mori R, Battistini L, Vigna E, Santini M, Loiaconi V, Pompilio G, Germani A, Capogrossi MC. Identification of myocardial and vascular precursor cells in human and mouse epicardium. *Circ Res.* 2007; 101: 1255-1265.

Lin H, McGrath JJ. Vasodilating effects of carbon monoxide. *Drug Chem Toxicol.* 1988; 11: 371-385.

Lin HH, Chen YH, Yet SF, Chau LY. After vascular injury, heme oxygenase-1/carbon monoxide enhances re-endothelialization via promoting mobilization of circulating endothelial progenitor cells. *J Thromb Haemost.* 2009; 7: 1401-1408.

Lin HH, Chen YH, Chang PF, Lee YT, Yet SF, Chau LY. Heme oxygenase-1 promotes neovascularization in ischemic heart by coinduction of VEGF and SDF-1. *J Mol Cell Cardiol.* 2008; 45: 44-55.

Lin LY, Kuo HK, Hwang JJ, Lai LP, Chiang FT, Tseng CD, Lin JL. Serum bilirubin is inversely associated with insulin resistance and metabolic syndrome among children and adolescents. *Atherosclerosis.* 2009; 203: 563-568.

Lin PH, Chiang MT, Chau LY. Ubiquitin-proteasome system mediates heme oxygenase-1 degradation through endoplasmic reticulum-associated degradation pathway. *Biochim Biophys Acta.* 2008; 1783: 1826-1834.

Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F, Dennery PA. Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *J Biol Chem.* 2007; 282: 20621-20633.

Lindenblatt N, Bordel R, Schareck W, Menger MD, Vollmar B. Vascular heme oxygenase-1 induction suppresses microvascular thrombus formation in vivo. *Arterioscler Thromb Vasc Biol.* 2004; 24: 601-606.

Link MS, Dockery DW. Air pollution and the triggering of cardiac arrhythmias. *Curr Opin Cardiol.* 2010; 25: 16-22.

Linke A, Muller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, Castaldo C, Cascapera S, Bohm M, Quaini F, Urbanek K, Leri A, Hintze TH, Kajstura J, Anversa P. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. *Proc Natl Acad Sci U S A.* 2005; 102: 8966-8971.

Liu X, Simpson JA, Brunt KR, Ward CA, Hall SR, Kinobe RT, Barrette V, Tse MY, Pang SC, Pachori AS, Dzau VJ, Ogunyankin KO, Melo LG. Preemptive heme oxygenase-1 gene delivery reveals reduced mortality and preservation of left ventricular function 1 yr after acute myocardial infarction. *Am J Physiol Heart Circ Physiol.* 2007; 293: H48-59.

Liu X, Pachori AS, Ward CA, Davis JP, Gnecchi M, Kong D, Zhang L, Murduck J, Yet SF, Perrella MA, Pratt RE, Dzau VJ, Melo LG. Heme oxygenase-1 (HO-1) inhibits postmyocardial infarct remodeling and restores ventricular function. *FASEB J.* 2006; 20: 207-216.

Liu X, Wei J, Peng DH, Layne MD, Yet SF. Absence of heme oxygenase-1 exacerbates myocardial ischemia/reperfusion injury in diabetic mice. *Diabetes.* 2005; 54: 778-784.

Liu XM, Chapman GB, Peyton KJ, Schafer AI, Durante W. Carbon monoxide inhibits apoptosis in vascular smooth muscle cells. *Cardiovasc Res.* 2002a; 55: 396-405.

Liu XM, Chapman GB, Wang H, Durante W. Adenovirus-mediated heme oxygenase-1 gene expression stimulates apoptosis in vascular smooth muscle cells. *Circulation.* 2002b; 105: 79-84.

Lo SS, Lin SC, Wu CW, Chen JH, Yeh WI, Chung MY, Lui WY. Heme oxygenase-1 gene promoter polymorphism is associated with risk of gastric adenocarcinoma and lymphovascular tumor invasion. *Ann Surg Oncol.* 2007; 14: 2250-2256.

Lublinghoff N, Winkler K, Winkelmann BR, Seelhorst U, Wellnitz B, Boehm BO, Marz W, Hoffmann MM. Genetic variants of the promoter of the heme oxygenase-1 gene and their influence on cardiovascular disease (the Ludwigshafen Risk and Cardiovascular Health study). *BMC Med Genet.* 2009; 10: 36.

Maghzal GJ, Leck MC, Collinson E, Li C, Stocker R. Limited role for the bilirubin-biliverdin redox amplification cycle in the cellular antioxidant protection by biliverdin reductase. *J Biol Chem.* 2009; 284: 29251-29259.

Maines MD. The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol.* 1997; 37: 517-554.

Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.* 1988; 2: 2557-2568.

Maines MD, Trakshel GM, Kutty RK. Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *J Biol Chem.* 1986; 261: 411-419.

Mancuso C, Bonsignore A, Di Stasio E, Mordente A, Motterlini R. Bilirubin and S-nitrosothiols interaction: evidence for a possible role of bilirubin as a scavenger of nitric oxide. *Biochem Pharmacol.* 2003; 66: 2355-2363.

Mansouri A, Perry CA. Alteration of platelet aggregation by cigarette smoke and carbon monoxide. *Thromb Haemost.* 1982; 48: 286-288.

Matsumoto H, Ishikawa K, Itabe H, Maruyama Y. Carbon monoxide and bilirubin from heme oxygenase-1 suppresses reactive oxygen species generation and plasminogen activator inhibitor-1 induction. *Mol Cell Biochem.* 2006; 291: 21-28.

Matsushita S, Kurihara H, Watanabe M, Okada T, Sakai T, Amano A. Alterations of phosphorylation state of connexin 43 during hypoxia and reoxygenation are associated with cardiac function. *J Histochem Cytochem.* 2006; 54: 343-353.

Matsushita T, Oyamada M, Fujimoto K, Yasuda Y, Masuda S, Wada Y, Oka T, Takamatsu T. Remodeling of cell-cell and cell-extracellular matrix interactions at the border zone of rat myocardial infarcts. *Circ Res.* 1999; 85: 1046-1055.

Maulik N, Sharma HS, Das DK. Induction of the haem oxygenase gene expression during the reperfusion of ischemic rat myocardium. *J Mol Cell Cardiol.* 1996; 28: 1261-1270.

Mazza F, Goodman A, Lombardo G, Vanella A, Abraham NG. Heme oxygenase-1 gene expression attenuates angiotensin II-mediated DNA damage in endothelial cells. *Exp Biol Med (Maywood).* 2003; 228: 576-583.

McCoubrey WK,Jr, Huang TJ, Maines MD. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem.* 1997; 247: 725-732.

Melley DD, Finney SJ, Elia A, Lagan AL, Quinlan GJ, Evans TW. Arterial carboxyhemoglobin level and outcome in critically ill patients. *Crit Care Med.* 2007; 35: 1882-1887.

Melo LG, Agrawal R, Zhang L, Rezvani M, Mangi AA, Ehsan A, Griese DP, Dell'Acqua G, Mann MJ, Oyama J, Yet SF, Layne MD, Perrella MA, Dzau VJ. Gene therapy strategy for long-term myocardial protection using adeno-associated virus-mediated delivery of heme oxygenase gene. *Circulation.* 2002; 105: 602-607.

Meyer G, Andre L, Tanguy S, Boissiere J, Farah C, Lopez-Lauri F, Gayrard S, Richard S, Boucher F, Cazorla O, Obert P, Reboul C. Simulated urban carbon monoxide air pollution exacerbates rat heart ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol.* 2010; 298: H1445-53.

Mito S, Ozono R, Oshima T, Yano Y, Watari Y, Yamamoto Y, Brydun A, Igarashi K, Yoshizumi M. Myocardial protection against pressure overload in mice lacking Bach1, a transcriptional repressor of heme oxygenase-1. *Hypertension.* 2008; 51: 1570-1577.

Miura T, Ohnuma Y, Kuno A, Tanno M, Ichikawa Y, Nakamura Y, Yano T, Miki T, Sakamoto J, Shimamoto K. Protective role of gap junctions in preconditioning against myocardial infarction. *Am J Physiol Heart Circ Physiol.* 2004; 286: H214-21.

Morimoto K, Ohta K, Yachie A, Yang Y, Shimizu M, Goto C, Toma T, Kasahara Y, Yokoyama H, Miyata T, Seki H, Koizumi S. Cytoprotective role of heme oxygenase (HO)-1 in human kidney with various renal diseases. *Kidney Int.* 2001; 60: 1858-1866.

Morita T, Mitsialis SA, Koike H, Liu Y, Kourembanas S. Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. *J Biol Chem.* 1997; 272: 32804-32809.

Morita T, Perrella MA, Lee ME, Kourembanas S. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc Natl Acad Sci U S A.* 1995; 92: 1475-1479.

Morse D, Pischke SE, Zhou Z, Davis RJ, Flavell RA, Loop T, Otterbein SL, Otterbein LE, Choi AM. Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J Biol Chem.* 2003; 278: 36993-36998.

Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med.* 2004; 10: 549-557.

Motterlini R, Foresti R, Bassi R, Calabrese V, Clark JE, Green CJ. Endothelial heme oxygenase-1 induction by hypoxia. Modulation by inducible nitric-oxide synthase and S-nitrosothiols. *J Biol Chem.* 2000a; 275: 13613-13620.

Motterlini R, Foresti R, Bassi R, Green CJ. Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. *Free Radic Biol Med.* 2000b; 28: 1303-1312.

Motterlini R, Foresti R, Intaglietta M, Winslow RM. NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *Am J Physiol.* 1996; 270: H107-14.

Musameh MD, Fuller BJ, Mann BE, Green CJ, Motterlini R. Positive inotropic effects of carbon monoxide-releasing molecules (CO-RMs) in the isolated perfused rat heart. *Br J Pharmacol.* 2006; 149: 1104-1112.

Mustafa S, Weltermann A, Fritsche R, Marsik C, Wagner O, Kyrle PA, Eichinger S. Genetic variation in heme oxygenase 1 (HMOX1) and the risk of recurrent venous thromboembolism. *J Vasc Surg.* 2008; 47: 566-570.

Nakamura H, Uetani Y, Komura M, Takada S, Sano K, Matsuo T. Inhibitory action of bilirubin on superoxide production by polymorphonuclear leukocytes. *Biol Neonate.* 1987; 52: 273-278.

Ndisang JF, Jadhav A. Upregulating the heme oxygenase system suppresses left ventricular hypertrophy in adult spontaneously hypertensive rats for 3 months. *J Card Fail.* 2009; 15: 616-628.

Ndisang JF, Wang R. Alterations in heme oxygenase/carbon monoxide system in pulmonary arteries in hypertension. *Exp Biol Med (Maywood).* 2003; 228: 557-563.

Ndisang JF, Zhao W, Wang R. Selective regulation of blood pressure by heme oxygenase-1 in hypertension. *Hypertension.* 2002; 40: 315-321.

Ning W, Song R, Li C, Park E, Mohsenin A, Choi AM, Choi ME. TGF-beta1 stimulates HO-1 via the p38 mitogen-activated protein kinase in A549 pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol.* 2002; 283: L1094-102.

Ockaili R, Natarajan R, Salloum F, Fisher BJ, Jones D, Fowler AA,3rd, Kukreja RC. HIF-1 activation attenuates postischemic myocardial injury: role for heme oxygenase-1 in modulating microvascular chemokine generation. *Am J Physiol Heart Circ Physiol.* 2005; 289: H542-8.

Ohlmann A, Giffhorn-Katz S, Becker I, Katz N, Immenschuh S. Regulation of heme oxygenase-1 gene expression by anoxia and reoxygenation in primary rat hepatocyte cultures. *Exp Biol Med (Maywood).* 2003; 228: 584-589.

Ohta K, Yachie A, Fujimoto K, Kaneda H, Wada T, Toma T, Seno A, Kasahara Y, Yokoyama H, Seki H, Koizumi S. Tubular injury as a cardinal pathologic feature in human heme oxygenase-1 deficiency. *Am J Kidney Dis.* 2000; 35: 863-870.

Ollinger R, Bilban M, Erat A, Froio A, McDaid J, Tyagi S, Csizmadia E, Graca-Souza AV, Liloia A, Soares MP, Otterbein LE, Usheva A, Yamashita K, Bach FH. Bilirubin: a natural inhibitor of vascular smooth muscle cell proliferation. *Circulation.* 2005; 112: 1030-1039.

Ono K, Goto Y, Takagi S, Baba S, Tago N, Nonogi H, Iwai N. A promoter variant of the heme oxygenase-1 gene may reduce the incidence of ischemic heart disease in Japanese. *Atherosclerosis.* 2004; 173: 315-319.

Ono K, Mannami T, Iwai N. Association of a promoter variant of the haeme oxygenase-1 gene with hypertension in women. *J Hypertens.* 2003; 21: 1497-1503.

Orozco LD, Kapturczak MH, Barajas B, Wang X, Weinstein MM, Wong J, Deshane J, Bolisetty S, Shaposhnik Z, Shih DM, Agarwal A, Lusis AJ, Araujo JA. Heme oxygenase-1 expression in macrophages plays a beneficial role in atherosclerosis. *Circ Res.* 2007; 100: 1703-1711.

Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol.* 2003a; 24: 449-455.

Otterbein LE, Zuckerbraun BS, Haga M, Liu F, Song R, Usheva A, Stachulak C, Bodyak N, Smith RN, Csizmadia E, Tyagi S, Akamatsu Y, Flavell RJ, Billiar TR, Tzeng E, Bach FH, Choi AM, Soares MP. Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. *Nat Med.* 2003b; 9: 183-190.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med.* 2000; 6: 422-428.

Otterbein LE, Choi AM. Heme oxygenase: colors of defense against cellular stress. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279: L1029-37.

Pabla R, Bland-Ward P, Moore PK, Curtis MJ. An endogenous protectant effect of cardiac cyclic GMP against reperfusion-induced ventricular fibrillation in the rat heart. *Br J Pharmacol.* 1995; 116: 2923-2930.

Pachori AS, Melo LG, Zhang L, Solomon SD, Dzau VJ. Chronic recurrent myocardial ischemic injury is significantly attenuated by pre-emptive adeno-associated virus heme oxygenase-1 gene delivery. *J Am Coll Cardiol.* 2006; 47: 635-643.

Pachori AS, Melo LG, Hart ML, Noiseux N, Zhang L, Morello F, Solomon SD, Stahl GL, Pratt RE, Dzau VJ. Hypoxia-regulated therapeutic gene as a preemptive treatment strategy against ischemia/reperfusion tissue injury. *Proc Natl Acad Sci U S A.* 2004; 101: 12282-12287.

Pae HO, Oh GS, Choi BM, Kim YM, Chung HT. A molecular cascade showing nitric oxide-heme oxygenase-1-vascular endothelial growth factor-interleukin-8 sequence in human endothelial cells. *Endocrinology.* 2005; 146: 2229-2238.

Panchenko MV, Farber HW, Korn JH. Induction of heme oxygenase-1 by hypoxia and free radicals in human dermal fibroblasts. *Am J Physiol Cell Physiol.* 2000; 278: C92-C101.

Pandol SJ, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: bench to the bedside. *Gastroenterology.* 2007; 132: 1127-1151.

Pellacani A, Wiesel P, Sharma A, Foster LC, Huggins GS, Yet SF, Perrella MA. Induction of heme oxygenase-1 during endotoxemia is downregulated by transforming growth factor-beta1. *Circ Res.* 1998; 83: 396-403.

Peng L, Mundada L, Stomel JM, Liu JJ, Sun J, Yet SF, Fay WP. Induction of heme oxygenase-1 expression inhibits platelet-dependent thrombosis. *Antioxid Redox Signal.* 2004; 6: 729-735.

Pepe M, Mamdani M, Zentilin L, Csiszar A, Qanud K, Zacchigna S, Ungvari Z, Puligadda U, Moimas S, Xu X, Edwards JG, Hintze TH, Giacca M, Recchia FA. Intramyocardial VEGF-B167 gene delivery delays the progression towards congestive failure in dogs with pacing-induced dilated cardiomyopathy. *Circ Res.* 2010; 106: 1893-1903.

Peterson SJ, Frishman WH, Abraham NG. Targeting heme oxygenase: therapeutic implications for diseases of the cardiovascular system. *Cardiol Rev.* 2009; 17: 99-111.

Petrache I, Otterbein LE, Alam J, Wiegand GW, Choi AM. Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. *Am J Physiol Lung Cell Mol Physiol.* 2000; 278: L312-9.

Peyton KJ, Reyna SV, Chapman GB, Ensenat D, Liu XM, Wang H, Schafer AI, Durante W. Heme oxygenase-1-derived carbon monoxide is an autocrine inhibitor of vascular smooth muscle cell growth. *Blood.* 2002; 99: 4443-4448.

Pfeffer MA, Pfeffer JM, Fishbein MC, Fletcher PJ, Spadaro J, Kloner RA, Braunwald E. Myocardial infarct size and ventricular function in rats. *Circ Res.* 1979; 44: 503-512.

Piantadosi CA, Carraway MS, Babiker A, Suliman HB. Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. *Circ Res.* 2008; 103: 1232-1240.

Plotkin LI, Manolagas SC, Bellido T. Transduction of cell survival signals by connexin-43 hemichannels. *J Biol Chem.* 2002; 277: 8648-8657.

Plotkin LI, Bellido T. Bisphosphonate-induced, hemichannel-mediated, anti-apoptosis through the Src/ERK pathway: a gap junction-independent action of connexin43. *Cell Commun Adhes.* 2001; 8: 377-382.

Pomeraniec Y, Grion N, Gadda L, Pannunzio V, Podesta EJ, Cymeryng CB. Adrenocorticotropin induces heme oxygenase-1 expression in adrenal cells. *J Endocrinol.* 2004; 180: 113-124.

Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. *Pharmacol Ther.* 2009; 123: 255-278.

Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci U S A.* 1997a; 94: 10925-10930.

Poss KD, Tonegawa S. Heme oxygenase 1 is required for mammalian iron reutilization. *Proc Natl Acad Sci U S A.* 1997b; 94: 10919-10924.

Purcell S, Cherny SS, Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics.* 2003; 19: 149-150.

Quist AP, Rhee SK, Lin H, Lal R. Physiological role of gap-junctional hemichannels. Extracellular calcium-dependent isosmotic volume regulation. *J Cell Biol.* 2000; 148: 1063-1074.

Raju VS, Imai N, Liang CS. Chamber-specific regulation of heme oxygenase-1 (heat shock protein 32) in right-sided congestive heart failure. *J Mol Cell Cardiol.* 1999; 31: 1581-1589.

Raju VS, Maines MD. Renal ischemia/reperfusion up-regulates heme oxygenase-1 (HSP32) expression and increases cGMP in rat heart. *J Pharmacol Exp Ther.* 1996; 277: 1814-1822.

Raju VS, Maines MD. Coordinated expression and mechanism of induction of HSP32 (heme oxygenase-1) mRNA by hyperthermia in rat organs. *Biochim Biophys Acta.* 1994; 1217: 273-280.

Redaelli CA, Tian YH, Schaffner T, Ledermann M, Baer HU, Dufour JF. Extended preservation of rat liver graft by induction of heme oxygenase-1. *Hepatology.* 2002; 35: 1082-1092.

Rizzardini M, Carelli M, Cabello Porras MR, Cantoni L. Mechanisms of endotoxin-induced haem oxygenase mRNA accumulation in mouse liver: synergism by glutathione depletion and protection by N-acetylcysteine. *Biochem J.* 1994; 304 (Pt 2): 477-483.

Rotenberg MO, Maines MD. Characterization of a cDNA-encoding rabbit brain heme oxygenase-2 and identification of a conserved domain among mammalian heme oxygenase isozymes: possible heme-binding site? *Arch Biochem Biophys.* 1991; 290: 336-344.

Rotenberg MO, Maines MD. Isolation, characterization, and expression in Escherichia coli of a cDNA encoding rat heme oxygenase-2. *J Biol Chem.* 1990; 265: 7501-7506.

Rucker M, Schafer T, Roesken F, Spitzer WJ, Bauer M, Menger MD. Reduction of inflammatory response in composite flap transfer by local stress conditioning-induced heat-shock protein 32. *Surgery.* 2001; 129: 292-301.

Rueda B, Oliver J, Robledo G, Lopez-Nevot MA, Balsa A, Pascual-Salcedo D, Gonzalez-Gay MA, Gonzalez-Escribano MF, Martin J. HO-1 promoter polymorphism associated with rheumatoid arthritis. *Arthritis Rheum.* 2007; 56: 3953-3958.

Rustad P, Simonsson P, Felding P, Pedersen M. Nordic Reference Interval Project Bio-bank and Database (NOBIDA): a source for future estimation and retrospective evaluation of reference intervals. *Scand J Clin Lab Invest.* 2004; 64: 431-438.

Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev.* 2006; 86: 583-650.

Sabaawy HE, Zhang F, Nguyen X, ElHosseiny A, Nasjletti A, Schwartzman M, Dennery P, Kappas A, Abraham NG. Human heme oxygenase-1 gene transfer lowers blood pressure and promotes growth in spontaneously hypertensive rats. *Hypertension.* 2001; 38: 210-215.

Sammut IA, Foresti R, Clark JE, Exon DJ, Vesely MJ, Sarathchandra P, Green CJ, Motterlini R. Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1. *Br J Pharmacol.* 1998; 125: 1437-1444.

Samuel SM, Thirunavukkarasu M, Penumathsa SV, Koneru S, Zhan L, Maulik G, Sudhakaran PR, Maulik N. Thioredoxin-1 gene therapy enhances angiogenic signaling and reduces ventricular remodeling in infarcted myocardium of diabetic rats. *Circulation.* 2010; 121: 1244-1255.

Sarady JK, Otterbein SL, Liu F, Otterbein LE, Choi AM. Carbon monoxide modulates endotoxin-induced production of granulocyte macrophage colony-stimulating factor in macrophages. *Am J Respir Cell Mol Biol.* 2002; 27: 739-745.

Saraste A, Pulkki K, Kallajoki M, Henriksen K, Parvinen M, Voipio-Pulkki LM. Apoptosis in human acute myocardial infarction. *Circulation.* 1997; 95: 320-323.

Sardana MK, Kappas A. Dual control mechanism for heme oxygenase: tin(IV)-protoporphyrin potently inhibits enzyme activity while markedly increasing content of enzyme protein in liver. *Proc Natl Acad Sci U S A.* 1987; 84: 2464-2468.

Sass G, Soares MC, Yamashita K, Seyfried S, Zimmermann WH, Eschenhagen T, Kaczmarek E, Ritter T, Volk HD, Tiegs G. Heme oxygenase-1 and its reaction product, carbon monoxide, prevent inflammation-related apoptotic liver damage in mice. *Hepatology.* 2003; 38: 909-918.

Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP. Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *J Immunol.* 2001; 166: 4185-4194.

Sato T, Takeno M, Honma K, Yamauchi H, Saito Y, Sasaki T, Morikubo H, Nagashima Y, Takagi S, Yamanaka K, Kaneko T, Ishigatsubo Y. Heme oxygenase-1, a potential biomarker of chronic silicosis, attenuates silica-induced lung injury. *Am J Respir Crit Care Med.* 2006; 174: 906-914.

Savio M, Stivala LA, Bianchi L, Vannini V, Prosperi E. Involvement of the proliferating cell nuclear antigen (PCNA) in DNA repair induced by alkylating agents and oxidative damage in human fibroblasts. *Carcinogenesis.* 1998; 19: 591-596.

Sawa T, Mounawar M, Tatemichi M, Gilibert I, Katoh T, Ohshima H. Increased risk of gastric cancer in Japanese subjects is associated with microsatellite polymorphisms in the heme oxygenase-1 and the inducible nitric oxide synthase gene promoters. *Cancer Lett.* 2008; 269: 78-84.

Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, Motterlini R. Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. *Mol Pharmacol.* 2002; 61: 554-561.

Scharte M, von Ostrowski TA, Daudel F, Freise H, Van Aken H, Bone HG. Endogenous carbon monoxide production correlates weakly with severity of acute illness. *Eur J Anaesthesiol.* 2006; 23: 117-122.

Schillinger M, Exner M, Minar E, Mlekusch W, Mullner M, Mannhalter C, Bach FH, Wagner O. Heme oxygenase-1 genotype and restenosis after balloon angioplasty: a novel vascular protective factor. *J Am Coll Cardiol.* 2004; 43: 950-957.

Schmidt R, Tritschler E, Hoetzel A, Loop T, Humar M, Halverscheid L, Geiger KK, Pannen BH. Heme oxygenase-1 induction by the clinically used anesthetic isoflurane protects rat livers from ischemia/reperfusion injury. *Ann Surg.* 2007; 245: 931-942.

Schulz R, Gres P, Skyschally A, Duschin A, Belosjorow S, Konietzka I, Heusch G. Ischemic preconditioning preserves connexin 43 phosphorylation during sustained ischemia in pig hearts in vivo. *FASEB J.* 2003; 17: 1355-1357.

Schwanke U, Konietzka I, Duschin A, Li X, Schulz R, Heusch G. No ischemic preconditioning in heterozygous connexin43-deficient mice. *Am J Physiol Heart Circ Physiol.* 2002; 283: H1740-2.

Schwer CI, Mutschler M, Stoll P, Goebel U, Humar M, Hoetzel A, Schmidt R. Carbon monoxide releasing molecule-2 inhibits pancreatic stellate cell proliferation by activating p38 mitogen-activated protein kinase/heme oxygenase-1 signaling. *Mol Pharmacol.* 2010; 77: 660-669.

Schwertner HA, Jackson WG, Tolan G. Association of low serum concentration of bilirubin with increased risk of coronary artery disease. *Clin Chem.* 1994; 40: 18-23.

Severs NJ, Bruce AF, Dupont E, Rothery S. Remodelling of gap junctions and connexin expression in diseased myocardium. *Cardiovasc Res.* 2008; 80: 9-19.

Severs NJ, Coppen SR, Dupont E, Yeh HI, Ko YS, Matsushita T. Gap junction alterations in human cardiac disease. *Cardiovasc Res.* 2004; 62: 368-377.

Sharma HS, Das DK, Verdouw PD. Enhanced expression and localization of heme oxygenase-1 during recovery phase of porcine stunned myocardium. *Mol Cell Biochem.* 1999; 196: 133-139.

Sheu CC, Zhai R, Wang Z, Gong MN, Tejera P, Chen F, Su L, Thompson BT, Christiani DC. Heme oxygenase-1 microsatellite polymorphism and haplotypes are associated with the development of acute respiratory distress syndrome. *Intensive Care Med.* 2009; 35: 1343-1351.

Shibahara S, Sato M, Muller RM, Yoshida T. Structural organization of the human heme oxygenase gene and the function of its promoter. *Eur J Biochem.* 1989; 179: 557-563.

Shibahara S, Muller RM, Taguchi H. Transcriptional control of rat heme oxygenase by heat shock. *J Biol Chem.* 1987; 262: 12889-12892.

Shibuya A, Onda K, Kawahara H, Uchiyama Y, Nakayama H, Omi T, Nagaoka M, Matsui H, Hirano T. Sofalcone, a gastric mucosa protective agent, increases vascular endothelial growth factor via the Nrf2-heme-oxygenase-1 dependent pathway in gastric epithelial cells. *Biochem Biophys Res Commun.* 2010; .

Sierra-Filardi E, Vega MA, Sanchez-Mateos P, Corbi AL, Puig-Kroger A. Heme Oxygenase-1 expression in M-CSF-polarized M2 macrophages contributes to LPS-induced IL-10 release. *Immunobiology.* 2010; .

Silva RF, Rodrigues CM, Brites D. Rat cultured neuronal and glial cells respond differently to toxicity of unconjugated bilirubin. *Pediatr Res.* 2002; 51: 535-541.

Smart N, Riley PR. The stem cell movement. *Circ Res.* 2008; 102: 1155-1168.

Smithline HA, Ward KR, Chiulli DA, Blake HC, Rivers EP. Whole body oxygen consumption and critical oxygen delivery in response to prolonged and severe carbon monoxide poisoning. *Resuscitation.* 2003; 56: 97-104.

Snider P, Standley KN, Wang J, Azhar M, Doetschman T, Conway SJ. Origin of cardiac fibroblasts and the role of periostin. *Circ Res.* 2009; 105: 934-947.

Soares MP, Seldon MP, Gregoire IP, Vassilevskaia T, Berberat PO, Yu J, Tsui TY, Bach FH. Heme oxygenase-1 modulates the expression of adhesion molecules associated with endothelial cell activation. *J Immunol.* 2004; 172: 3553-3563.

Soares MP, Usheva A, Brouard S, Berberat PO, Gunther L, Tobiasch E, Bach FH. Modulation of endothelial cell apoptosis by heme oxygenase-1-derived carbon monoxide. *Antioxid Redox Signal.* 2002; 4: 321-329.

Solan JL, Lampe PD. Connexin43 phosphorylation: structural changes and biological effects. *Biochem J.* 2009; 419: 261-272.

Song F, Li X, Zhang M, Yao P, Yang N, Sun X, Hu FB, Liu L. Association between heme oxygenase-1 gene promoter polymorphisms and type 2 diabetes in a Chinese population. *Am J Epidemiol.* 2009a; 170: 747-756.

Song H, Bergstrasser C, Rafat N, Hoger S, Schmidt M, Endres N, Goebeler M, Hillebrands JL, Brigelius-Flohe R, Banning A, Beck G, Loesel R, Yard BA. The carbon monoxide releasing molecule (CORM-3) inhibits expression of vascular cell adhesion molecule-1 and E-selectin independently of haem oxygenase-1 expression. *Br J Pharmacol.* 2009b; 157: 769-780.

Song R, Mahidhara RS, Zhou Z, Hoffman RA, Seol DW, Flavell RA, Billiar TR, Otterbein LE, Choi AM. Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. *J Immunol.* 2004; 172: 1220-1226.

Song R, Kubo M, Morse D, Zhou Z, Zhang X, Dauber JH, Fabisiak J, Alber SM, Watkins SC, Zuckerbraun BS, Otterbein LE, Ning W, Oury TD, Lee PJ, McCurry KR, Choi AM. Carbon monoxide induces cytoprotection in rat orthotopic lung transplantation via anti-inflammatory and anti-apoptotic effects. *Am J Pathol.* 2003; 163: 231-242.

Song R, Mahidhara RS, Liu F, Ning W, Otterbein LE, Choi AM. Carbon monoxide inhibits human airway smooth muscle cell proliferation via mitogen-activated protein kinase pathway. *Am J Respir Cell Mol Biol.* 2002; 27: 603-610.

Srinivas G, Anversa P, Frishman WH. Cytokines and myocardial regeneration: a novel treatment option for acute myocardial infarction. *Cardiol Rev.* 2009; 17: 1-9.

Stein AB, Guo Y, Tan W, Wu WJ, Zhu X, Li Q, Luo C, Dawn B, Johnson TR, Motterlini R, Bolli R. Administration of a CO-releasing molecule induces late preconditioning against myocardial infarction. *J Mol Cell Cardiol.* 2005; 38: 127-134.

Stocker R. Antioxidant activities of bile pigments. *Antioxid Redox Signal.* 2004; 6: 841-849.

Stocker R, Peterhans E. Antioxidant properties of conjugated bilirubin and biliverdin: biologically relevant scavenging of hypochlorous acid. *Free Radic Res Commun.* 1989; 6: 57-66.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science.* 1987; 235: 1043-1046.

Sun J, Brand M, Zenke Y, Tashiro S, Groudine M, Igarashi K. Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. *Proc Natl Acad Sci U S A.* 2004; 101: 1461-1466.

Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J, Taketo MM, Yamamoto M, Igarashi K. Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J.* 2002; 21: 5216-5224.

Sun Y, Weber KT. Infarct scar: a dynamic tissue. *Cardiovasc Res.* 2000; 46: 250-256.

Suzuki H, Tashiro S, Hira S, Sun J, Yamazaki C, Zenke Y, Ikeda-Saito M, Yoshida M, Igarashi K. Heme regulates gene expression by triggering Crm1-dependent nuclear export of Bach1. *EMBO J.* 2004; 23: 2544-2553.

Suzuki M, Iso-o N, Takeshita S, Tsukamoto K, Mori I, Sato T, Ohno M, Nagai R, Ishizaka N. Facilitated angiogenesis induced by heme oxygenase-1 gene transfer in a rat model of hindlimb ischemia. *Biochem Biophys Res Commun.* 2003; 302: 138-143.

Sylvester JT, McGowan C. The effects of agents that bind to cytochrome P-450 on hypoxic pulmonary vasoconstriction. *Circ Res.* 1978; 43: 429-437.

Taha H, Skrzypek K, Guevara I, Nigisch A, Mustafa S, Grochot-Przeczek A, Ferdek P, Was H, Kotlinowski J, Kozakowska M, Balcerczyk A, Muchova L, Vitek L, Weigel G, Dulak J, Jozkowicz A. Role of heme oxygenase-1 in human endothelial cells: lesson from the promoter allelic variants. *Arterioscler Thromb Vasc Biol.* 2010; 30: 1634-1641.

Taketani S, Kohno H, Yoshinaga T, Tokunaga R. The human 32-kDa stress protein induced by exposure to arsenite and cadmium ions is heme oxygenase. *FEBS Lett.* 1989; 245: 173-176.

Taketani S, Kohno H, Yoshinaga T, Tokunaga R. Induction of heme oxygenase in rat hepatoma cells by exposure to heavy metals and hyperthermia. *Biochem Int.* 1988; 17: 665-672.

Tanaka M, Fukui M, Tomiyasu K, Akabame S, Nakano K, Hasegawa G, Oda Y, Nakamura N. Low serum bilirubin concentration is associated with coronary artery calcification (CAC). *Atherosclerosis.* 2009; 206: 287-291.

Tang YL, Tang Y, Zhang YC, Qian K, Shen L, Phillips MI. Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. *J Am Coll Cardiol.* 2005; 46: 1339-1350.

Tenhunen R, Marver HS, Schmid R. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J Lab Clin Med.* 1970a; 75: 410-421.

Tenhunen R, Ross ME, Marver HS, Schmid R. Reduced nicotinamide-adenine dinucleotide phosphate dependent biliverdin reductase: partial purification and characterization. *Biochemistry.* 1970b; 9: 298-303.

Tenhunen R, Marver HS, Schmid R. Microsomal heme oxygenase. Characterization of the enzyme. *J Biol Chem.* 1969; 244: 6388-6394.

Tenhunen R, Marver HS, Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci U S A.* 1968; 61: 748-755.

Teran FJ, Johnson RA, Stevenson BK, Peyton KJ, Jackson KE, Appleton SD, Durante W, Johnson FK. Heme oxygenase-derived carbon monoxide promotes arteriolar endothelial dysfunction and contributes to salt-induced hypertension in Dahl salt-sensitive rats. *Am J Physiol Regul Integr Comp Physiol.* 2005; 288: R615-22.

Terry CM, Clikeman JA, Hoidal JR, Callahan KS. TNF-alpha and IL-1alpha induce heme oxygenase-1 via protein kinase C, Ca2+, and phospholipase A2 in endothelial cells. *Am J Physiol.* 1999; 276: H1493-501.

Terry CM, Clikeman JA, Hoidal JR, Callahan KS. Effect of tumor necrosis factor-alpha and interleukin-1 alpha on heme oxygenase-1 expression in human endothelial cells. *Am J Physiol.* 1998; 274: H883-91.

Thom SR, Fisher D, Xu YA, Notarfrancesco K, Ischiropoulos H. Adaptive responses and apoptosis in endothelial cells exposed to carbon monoxide. *Proc Natl Acad Sci U S A.* 2000; 97: 1305-1310.

Thorup C, Jones CL, Gross SS, Moore LC, Goligorsky MS. Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS. *Am J Physiol.* 1999; 277: F882-9.

Tillmanns J, Rota M, Hosoda T, Misao Y, Esposito G, Gonzalez A, Vitale S, Parolin C, Yasuzawa-Amano S, Muraski J, De Angelis A, Lecapitaine N, Siggins RW, Loredo M, Bearzi C, Bolli R, Urbanek K, Leri A, Kajstura J, Anversa P. Formation of large coronary arteries by cardiac progenitor cells. *Proc Natl Acad Sci U S A.* 2008; 105: 1668-1673.

Tiroch K, Koch W, von Beckerath N, Kastrati A, Schomig A. Heme oxygenase-1 gene promoter polymorphism and restenosis following coronary stenting. *Eur Heart J.* 2007; 28: 968-973.

Tomanek RJ, Ishii Y, Holifield JS, Sjogren CL, Hansen HK, Mikawa T. VEGF family members regulate myocardial tubulogenesis and coronary artery formation in the embryo. *Circ Res.* 2006; 98: 947-953.

Tomanek RJ, Holifield JS, Reiter RS, Sandra A, Lin JJ. Role of VEGF family members and receptors in coronary vessel formation. *Dev Dyn.* 2002; 225: 233-240.

Tongers J, Knapp JM, Korf M, Kempf T, Limbourg A, Limbourg FP, Li Z, Fraccarollo D, Bauersachs J, Han X, Drexler H, Fiedler B, Wollert KC. Haeme oxygenase promotes progenitor cell mobilization, neovascularization, and functional recovery after critical hindlimb ischaemia in mice. *Cardiovasc Res.* 2008; 78: 294-300.

True AL, Olive M, Boehm M, San H, Westrick RJ, Raghavachari N, Xu X, Lynn EG, Sack MN, Munson PJ, Gladwin MT, Nabel EG. Heme oxygenase-1 deficiency accelerates formation of arterial thrombosis through oxidative damage to the endothelium, which is rescued by inhaled carbon monoxide. *Circ Res.* 2007; 101: 893-901.

Tsubokawa T, Yagi K, Nakanishi C, Zuka M, Nohara A, Ino H, Fujino N, Konno T, Kawashiri MA, Ishibashi-Ueda H, Nagaya N, Yamagishi M. Impact of anti-apoptotic and anti-oxidative effects of bone marrow mesenchymal stem cells with transient overexpression of heme oxygenase-1 on myocardial ischemia. *Am J Physiol Heart Circ Physiol.* 2010; 298: H1320-9.

Tsuburai T, Suzuki M, Nagashima Y, Suzuki S, Inoue S, Hasiba T, Ueda A, Ikehara K, Matsuse T, Ishigatsubo Y. Adenovirus-mediated transfer and overexpression of heme oxygenase 1 cDNA in lung prevents bleomycin-induced pulmonary fibrosis via a Fas-Fas ligand-independent pathway. *Hum Gene Ther.* 2002; 13: 1945-1960.

Tsui TY, Lau CK, Ma J, Wu X, Wang YQ, Farkas S, Xu R, Schlitt HJ, Fan ST. rAAV-mediated stable expression of heme oxygenase-1 in stellate cells: a new approach to attenuate liver fibrosis in rats. *Hepatology.* 2005; 42: 335-342.

Turpeinen H, Kyllonen LE, Parkkinen J, Laine J, Salmela KT, Partanen J. Heme oxygenase 1 gene polymorphisms and outcome of renal transplantation. *Int J Immunogenet.* 2007; 34: 253-257.

Uemura K, Adachi-Akahane S, Shintani-Ishida K, Yoshida K. Carbon monoxide protects cardiomyogenic cells against ischemic death through L-type Ca2+ channel inhibition. *Biochem Biophys Res Commun.* 2005; 334: 661-668.

Ullrich R, Exner M, Schillinger M, Zuckermann A, Raith M, Dunkler D, Horvat R, Grimm M, Wagner O. Microsatellite polymorphism in the heme oxygenase-1 gene promoter and cardiac allograft vasculopathy. *J Heart Lung Transplant.* 2005; 24: 1600-1605.

van Tuyn J, Atsma DE, Winter EM, van der Velde-van Dijke I, Pijnappels DA, Bax NA, Knaan-Shanzer S, Gittenberger-de Groot AC, Poelmann RE, van der Laarse A, van der Wall EE, Schalij MJ, de Vries AA. Epicardial cells of human adults can undergo an epithelial-to-mesenchymal transition and obtain characteristics of smooth muscle cells in vitro. *Stem Cells.* 2007; 25: 271-278.

Varadi J, Lekli I, Juhasz B, Bacskay I, Szabo G, Gesztelyi R, Szendrei L, Varga E, Bak I, Foresti R, Motterlini R, Tosaki A. Beneficial effects of carbon monoxide-releasing molecules on post-ischemic myocardial recovery. *Life Sci.* 2007; 80: 1619-1626.

Vetterlein F, Muhlfeld C, Cetegen C, Volkmann R, Schrader C, Hellige G. Redistribution of connexin43 in regional acute ischemic myocardium: influence of ischemic preconditioning. *Am J Physiol Heart Circ Physiol.* 2006; 291: H813-9.

Vile GF, Tyrrell RM. Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. *J Biol Chem.* 1993; 268: 14678-14681.

Vincent JL, de Mendonca A, Cantraine F, Moreno R, Takala J, Suter PM, Sprung CL, Colardyn F, Blecher S. Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicenter, prospective study. Working group on "sepsis-related problems" of the European Society of Intensive Care Medicine. *Crit Care Med.* 1998; 26: 1793-1800.

Vitek L, Jirsa M, Brodanova M, Kalab M, Marecek Z, Danzig V, Novotny L, Kotal P. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. *Atherosclerosis.* 2002; 160: 449-456.

Vulapalli SR, Chen Z, Chua BH, Wang T, Liang CS. Cardioselective overexpression of HO-1 prevents I/R-induced cardiac dysfunction and apoptosis. *Am J Physiol Heart Circ Physiol.* 2002; 283: H688-94.

Wada AM, Smith TK, Osler ME, Reese DE, Bader DM. Epicardial/Mesothelial cell line retains vasculogenic potential of embryonic epicardium. *Circ Res.* 2003; 92: 525-531.

Wagner CT, Durante W, Christodoulides N, Hellums JD, Schafer AI. Hemodynamic forces induce the expression of heme oxygenase in cultured vascular smooth muscle cells. *J Clin Invest.* 1997; 100: 589-596.

Wang G, Hamid T, Keith RJ, Zhou G, Partridge CR, Xiang X, Kingery JR, Lewis RK, Li Q, Rokosh DG, Ford R, Spinale FG, Riggs DW, Srivastava S, Bhatnagar A, Bolli R, Prabhu SD. Cardioprotective and antiapoptotic effects of heme oxygenase-1 in the failing heart. *Circulation.* 2010; 121: 1912-1925.

Wang L, Lee JY, Kwak JH, He Y, Kim SI, Choi ME. Protective effects of low-dose carbon monoxide against renal fibrosis induced by unilateral ureteral obstruction. *Am J Physiol Renal Physiol.* 2008; 294: F508-17.

Wang LJ, Lee TS, Lee FY, Pai RC, Chau LY. Expression of heme oxygenase-1 in atherosclerotic lesions. *Am J Pathol.* 1998; 152: 711-720.

Wang R, Shamloul R, Wang X, Meng Q, Wu L. Sustained normalization of high blood pressure in spontaneously hypertensive rats by implanted hemin pump. *Hypertension.* 2006; 48: 685-692.

Wang R, Wang Z, Wu L. Carbon monoxide-induced vasorelaxation and the underlying mechanisms. *Br J Pharmacol.* 1997; 121: 927-934.

Wang R, Wu L. The chemical modification of KCa channels by carbon monoxide in vascular smooth muscle cells. *J Biol Chem.* 1997; 272: 8222-8226.

Wegiel B, Gallo DJ, Raman KG, Karlsson JM, Ozanich B, Chin BY, Tzeng E, Ahmad S, Ahmed A, Baty CJ, Otterbein LE. Nitric oxide-dependent bone marrow progenitor mobilization by carbon monoxide enhances endothelial repair after vascular injury. *Circulation.* 2010; 121: 537-548.

Wegiel B, Chin BY, Otterbein LE. Inhale to survive, cycle or die? Carbon monoxide and cellular proliferation. *Cell Cycle.* 2008; 7: 1379-1384.

Weidemann A, Johnson RS. Biology of HIF-1alpha. *Cell Death Differ.* 2008; 15: 621-627.

Weis N, Weigert A, von Knethen A, Brune B. Heme oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. *Mol Biol Cell.* 2009; 20: 1280-1288.

Wiesel P, Patel AP, Carvajal IM, Wang ZY, Pellacani A, Maemura K, DiFonzo N, Rennke HG, Layne MD, Yet SF, Lee ME, Perrella MA. Exacerbation of chronic renovascular hypertension and acute renal failure in heme oxygenase-1-deficient mice. *Circ Res.* 2001; 88: 1088-1094.

Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. *Nat Med.* 1996; 2: 87-90.

Winter EM, Gittenberger-de Groot AC. Epicardium-derived cells in cardiogenesis and cardiac regeneration. *Cell Mol Life Sci.* 2007; 64: 692-703.

Woo YJ, Panlilio CM, Cheng RK, Liao GP, Atluri P, Hsu VM, Cohen JE, Chaudhry HW. Therapeutic delivery of cyclin A2 induces myocardial regeneration and enhances cardiac function in ischemic heart failure. *Circulation.* 2006; 114: I206-13.

Wu BJ, Midwinter RG, Cassano C, Beck K, Wang Y, Changsiri D, Gamble JR, Stocker R. Heme oxygenase-1 increases endothelial progenitor cells. *Arterioscler Thromb Vasc Biol.* 2009; 29: 1537-1542.

Wu TW, Fung KP, Yang CC. Unconjugated bilirubin inhibits the oxidation of human low density lipoprotein better than Trolox. *Life Sci.* 1994; 54: P477-81.

Xia ZW, Xu LQ, Zhong WW, Wei JJ, Li NL, Shao J, Li YZ, Yu SC, Zhang ZL. Heme oxygenase-1 attenuates ovalbumin-induced airway inflammation by up-regulation of foxp3 T-regulatory cells, interleukin-10, and membrane-bound transforming growth factor- 1. *Am J Pathol.* 2007; 171: 1904-1914.

Yachie A, Toma T, Mizuno K, Okamoto H, Shimura S, Ohta K, Kasahara Y, Koizumi S. Heme oxygenase-1 production by peripheral blood monocytes during acute inflammatory illnesses of children. *Exp Biol Med (Maywood).* 2003; 228: 550-556.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest.* 1999; 103: 129-135.

Yamada N, Yamaya M, Okinaga S, Nakayama K, Sekizawa K, Shibahara S, Sasaki H. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. *Am J Hum Genet.* 2000; 66: 187-195.

Yang G, Nguyen X, Ou J, Rekulapelli P, Stevenson DK, Dennery PA. Unique effects of zinc protoporphyrin on HO-1 induction and apoptosis. *Blood.* 2001; 97: 1306-1313.

Yang L, Quan S, Nasjletti A, Laniado-Schwartzman M, Abraham NG. Heme oxygenase-1 gene expression modulates angiotensin II-induced increase in blood pressure. *Hypertension.* 2004; 43: 1221-1226.

Yasuda H, Okinaga S, Yamaya M, Ohrui T, Higuchi M, Shinkawa M, Itabashi S, Nakayama K, Asada M, Kikuchi A, Shibahara S, Sasaki H. Association of susceptibility to the development of pneumonia in the older Japanese population with haem oxygenase-1 gene promoter polymorphism. *J Med Genet.* 2006; 43: e17.

Yet SF, Layne MD, Liu X, Chen YH, Ith B, Sibinga NE, Perrella MA. Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *FASEB J.* 2003; 17: 1759-1761.

Yet SF, Tian R, Layne MD, Wang ZY, Maemura K, Solovyeva M, Ith B, Melo LG, Zhang L, Ingwall JS, Dzau VJ, Lee ME, Perrella MA. Cardiac-specific expression of heme oxygenase-1 protects against ischemia and reperfusion injury in transgenic mice. *Circ Res.* 2001; 89: 168-173.

Yet SF, Perrella MA, Layne MD, Hsieh CM, Maemura K, Kobzik L, Wiesel P, Christou H, Kourembanas S, Lee ME. Hypoxia induces severe right ventricular dilatation and infarction in heme oxygenase-1 null mice. *J Clin Invest.* 1999; 103: R23-9.

Yoshida T, Biro P, Cohen T, Muller RM, Shibahara S. Human heme oxygenase cDNA and induction of its mRNA by hemin. *Eur J Biochem.* 1988; 171: 457-461.

Yuasa S, Fukuda K, Tomita Y, Fujita J, Ieda M, Tahara S, Itabashi Y, Yagi T, Kawaguchi H, Hisaka Y, Ogawa S. Cardiomyocytes undergo cells division following myocardial infarction is a spatially and temporally restricted event in rats. *Mol Cell Biochem.* 2004; 259: 177-181.

Zeng B, Chen H, Zhu C, Ren X, Lin G, Cao F. Effects of combined mesenchymal stem cells and heme oxygenase-1 therapy on cardiac performance. *Eur J Cardiothorac Surg.* 2008a; 34: 850-856.

Zeng B, Ren X, Lin G, Zhu C, Chen H, Yin J, Jiang H, Yang B, Ding D. Paracrine action of HO-1-modified mesenchymal stem cells mediates cardiac protection and functional improvement. *Cell Biol Int.* 2008b; 32: 1256-1264.

Zhang F, Tang Z, Hou X, Lennartsson J, Li Y, Koch AW, Scotney P, Lee C, Arjunan P, Dong L, Kumar A, Rissanen TT, Wang B, Nagai N, Fons P, Fariss R, Zhang Y, Wawrousek E, Tansey G, Raber J, Fong GH, Ding H, Greenberg DA, Becker KG, Herbert JM, Nash A, Yla-Herttuala S, Cao Y, Watts RJ, Li X. VEGF-B is dispensable for blood vessel growth but critical for their survival, and VEGF-B targeting inhibits pathological angiogenesis. *Proc Natl Acad Sci U S A.* 2009; 106: 6152-6157.

Zhang K, Calabrese P, Nordborg M, Sun F. Haplotype block structure and its applications to association studies: power and study designs. *Am J Hum Genet.* 2002; 71: 1386-1394.

Zhang S, Lu S, Ge J, Guo J, Chen P, Li T, Zhang P, Jia Z, Ma K, Liu Y, Zhou C, Li L. Increased heme oxygenase-1 expression in infarcted rat hearts following human bone marrow mesenchymal cell transplantation. *Microvasc Res.* 2005; 69: 64-70.

Zhao T, Zhang D, Millard RW, Ashraf M, Wang Y. Stem cell homing and angiomyogenesis in transplanted hearts are enhanced by combined intramyocardial SDF-1alpha delivery and endogenous cytokine signaling. *Am J Physiol Heart Circ Physiol.* 2009; 296: H976-86.

Zhou Z, Song R, Fattman CL, Greenhill S, Alber S, Oury TD, Choi AM, Morse D. Carbon monoxide suppresses bleomycin-induced lung fibrosis. *Am J Pathol.* 2005; 166: 27-37.